CHARACTERIZING THE ROLES OF SUMOYLATION IN MITOSIS THROUGH SUBSTRATE IDENTIFICATION AND AN ANALYSIS OF THE SUMO ISOPEPTIDASES, SENP1 AND SENP2

by
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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
October, 2013

ABSTRACT

Cell cycle regulation is essential for all organisms and has profound impacts on development, tissue regeneration, and when aberrant, results in cancer. Mitosis, the process of nuclear division, is highly regulated by posttranslational modifications including sumoylation. The SUMO family consists of three paralogs, SUMO-1, -2 and -3, which are covalently attached to the lysine residues of substrates and affects substrate localization, function, and/or protein-protein interactions. In this thesis, we hypothesized that dynamic SUMO modification and demodification is essential for mitotic progression and that deregulation of the sumoylation machinery can lead to mitotic defects resulting in cancer. We analyzed the function of sumoylation in mitosis by a two-pronged approach. First, we conducted a mass spectrometry study to identify the proteins sumoylated in mitosis, which will provide a foundation for future studies identifying the molecular mechanisms of mitotic SUMO functions. Secondly, we characterized two SUMO deconjugating enzymes, SENP1 and SENP2, to enhance our understanding for how sumoylation is regulated temporally and spatially in mitosis. We demonstrated that sumoylation is required for chromosome alignment through SENP2 overexpression studies. Furthermore, we demonstrated that desumoylation is required for a timely metaphase to anaphase transition through SENP1 siRNA knockdown analysis. Finally, we conducted a literature review to describe the functions of sumoylation in regulating chromatin structure, which may impact the mitotic functions of sumoylation in chromosome condensation and/or centromere structure. In its entirety, this thesis presents a foundation of mitotic SUMO substrates for further analysis and a mechanism of isopeptidase-mediated regulation of sumoylation in mitosis.

Thesis Advisor: Dr. Michael J. Matunis

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Alternate Thesis Readers: Dr. Philip Jordan and Dr. Michael Trush

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Matunis for his advisement and mentorship during my graduate career. The production and writing of this thesis would not have been possible without his help. I would specifically like to thank Mike for helping me develop a number of skills that I believe will be critical in my future career as a scientist. Mike has taught me how to break down complicated scientific stories into very clean and concise presentations and has dramatically improved my scientific writing through his helpful suggestions and edits. I am so thankful that I have learned how to effectively communicate science in these two ways through his mentorship. Finally, I would like to thank him for all of his helpful discussions about my project. I have dramatically improved my ability to develop and design well-controlled experiments through these discussions.

In addition, I would like to thank the Hopkins community at large for all of their support over the years. The faculty of the BMB department has always been willing to take time out of their busy schedules to troubleshoot experiments and offer suggestions for better reagents. I have really enjoyed being part of such a positive and collaborative environment. In addition, I would like to specifically thank my thesis committee members and thesis readers who have given me many helpful suggestions over the years including Dr. Janice Evans, Dr. Valeria Culotta, Dr. Yixian Zheng, Dr. Gary Ketner, Dr. Michael Trush and Dr. Philip Jordan. Furthermore, I would like to thank the BMB staff, particularly Sharon Warner. Your work helps the BMB program run effortlessly and smoothly and I appreciate all the assistance you have given me over the years. Finally, I would like to thank all of the members of the Matunis lab, past and present. I learned a

tremendous amount about science through my interactions with you and had a lot of fun in the process. I hope that we all stay in touch for many years to come. You are all great scientists and I wish you the best of luck in the future.

Finally, I would like to thank my family and friends for all of their support during graduate school. Learning how to become an independent researcher involves a lot of ups and downs, and you all were there for me during the whole process. I could not have made it through this process without you and saying thank you does not even begin to express how much I have appreciated your support.

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ABBREVIATIONS AND NOMENCLATURE

ALT	alternate lengthening of telomeres
APB	ALT-associated PML nuclear bodies
APC	anaphase promoting complex
ATP	adenosine triphosphate
BER	base excision repair
C. elegans	*Caenorhabditis elegans*
CAT	catalytic domain
CCAN	constitutive centromere associated network
chIP	chromatin immunoprecipitation
CO_2	carbon dioxide
CPC	chromosome passenger complex
CpG	cystosine-phosphate-guanine
D. melanogaster	*Drosophila melanogaster*
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNMT	deoxyribonucleic acid methyltransferase
E1	SUMO activating enzyme
E2	SUMO conjugation enzyme
E3	ubiquitin or SUMO ligase enzyme
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting

FKBP	FK506 binding protein
FRB	FKBP-rapamycin-associated protein
GFP	green fluorescent protein
GG	di-glycine
GST	glutathione S-transferase
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HP1α	heterochromatin protein 1α
HPLC	High-performance liquid chromatography
Hsp90	heat shock protein 90
kDa	kiloDalton
M	molar
MBD	methyl-cytosine-phosphate-guanine binding domain
MEFs	mouse embryonic fibroblasts
mL	milliliter
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NEM	N-ethylmaleimide
NES	nuclear export signal
nLC-ESI-MS/MS	nanoflow liquid chromatography electrospray ionization tandem mass spectrometry
NLS	nuclear localization signal
NPC	nuclear pore complex

Nup	nucleoporin
PBS	phosphate-buffered saline
PcG	Polycomb group
PIAS	protein inhibitor of activated STAT
PML	promyelocytic leukemia
PMSF	phenylmethanesulfonylfluoride
PRC	Polycomb repressive complex
PTM	post-translational modification
rDNA	ribosomal deoxyribonucleic acid
RING	really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal ribonucleic acid
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Saccharomyces pombe*
SAC	spindle assembly checkpoint
SAINT	probabilistic scoring of affinity purification-mass spectrometry data
SCM	sex comb on midleg
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SENP	sentrin-specific protease
SIM	SUMO interaction motif

siRNA	short, interfering RNA
STUbL	SUMO-targeted ubiquitin ligase
SUMO	small ubiquitin-related modifier
TDG	thymidine DNA glycosylase
TNX-100	triton-X 100
Tris	tris(hydroxymethyl)aminomethane
USPL1	ubiquitin-specific protease-like protein 1
X. laevis	*Xenopus laevis*
µg	microgram
µl	microliter
µm	micrometer
YFP	yellow fluorescent protein

CHAPTER 1

SUMOYLATION: A POSTTRANSLATIONAL REGULTOR OF MITOSIS

ABSTRACT

Mitosis is highly regulated by posttranslational modifications (PTMs), including phosphorylation, ubiquitination, and sumoylation. Further characterizing how mitosis is regulated is critical for enhancing our knowledge about mitotic aberrant events like cancer cell division. In this chapter, I will focus on sumoylation and explain how this PTM occurs and functions in the cell. Then, I will summarize the studies demonstrating that small ubiquitin-related modifiers (SUMOs) regulate mitosis at multiple stages and through a variety of mechanisms.

MAIN TEXT

Post-translational modifications and cell cycle regulation

Cell cycle regulation is essential for all organisms and has profound impacts on development, tissue regeneration, and when aberrant, results in cancer. Post-translational modifications (PTMs) are a hallmark of cell cycle regulation because of their highly dynamic nature and ability to alter protein function. Since the discovery of the cyclin proteins, ubiquitination and phosphorylation have been shown to be critical regulators of the cell cycle. In particular, mitosis, the phase that ensures high fidelity chromosome segregation between mother and daughter cells, is highly regulated by PTMs. Phosphorylation occurs early in mitosis to promote chromosome alignment. At the metaphase to anaphase transition, rapid dephosphoylation and ubiquitination occurs to initiate turnover of the mitosis specific proteins and reset the mitotic state. However, in recent years, the small ubiquitin-related modifier (SUMO) has also been demonstrated to play a key role in mitotic regulation, which will be the focus of this chapter.

SUMO Family and the Function of the SUMO signal

The small ubiquitin-related modifier (SUMO), like ubiquitin, is conjugated to other proteins through an isopeptide linkage between the carboxyl-terminus of SUMO and the lysine residue of a target protein. Both ubiquitin and SUMO are synthesized as precursor proteins that must be cleaved by isopeptidases to expose a C-terminal GG-motif, which makes the proteins functional for conjugation (1). In vertebrates, four different SUMO paralogs have been identified in the genome: SUMO-1, SUMO-2, SUMO-3, and SUMO-4 (Figure 1A). However, SUMO-4 contains a non-conserved

proline residue that prevents precursor processing into the mature form and thus cannot function as a PTM (2). SUMO-2 and -3 share ~97% identity and cannot be distinguished with current tools and are thus often referred to collectively as SUMO-2/3. In contrast, SUMO-1 only shares ~50% identity with SUMO-2/3 and is functionally unique from the other two paralogs (3). For example, SUMO-1 but not SUMO-2 can complement a yeast strain deficient in yeast SUMO, known as *smt3* (H. Newman, unpublished). Furthermore, the different SUMO paralogs have unique localizations and modify unique substrates in interphase and mitosis (Figure 1B) (4). Thus, SUMO-1 and SUMO-2/3 and their characterized functions in mitosis will be the focus on this chapter.

SUMO generates unique signals through the unique paralogs, monosumoylation, polysumoylation, and a combination of sumoylation and other PTMs (Figure 1C). SUMO-1 and SUMO-2/3 are conjugated to distinct pools of protein substrates. For example, SUMO-1 is required for proper Ran GTPase-activating protein (RanGAP1) targeting to the nuclear pore complex (NPC) (5), while SUMO-2/3 is required for CENP-E targeting to the kinetochore (4). Furthermore, SUMO can form polymeric chains that act as another unique signal, which is illustrated by preferential binding of RNF4 to polymeric SUMO-2 (6). Finally, there is now evidence that hybrid SUMO-ubiquitin chains are important in the recruitment of DNA repair factors to sites of DNA damage, further illustrating how combinations of PTMs can further diversify the SUMO signals (7).

Once a protein has been modified by SUMO, it can interact with proteins containing a SUMO-interacting motif (SIM). The best dcharacterized SIM is a hydrophobic patch (V/I-X-V/I-V/I) with a flanking acidic patch (8). However, other non-

consensus SIMs have been discovered, like the noncanonical SIM in CoREST1, so other novel SIMs are likely to be characterized in the future (9). Thus, once a protein is sumoylated it can be recognized by proteins containing SIMs, and more specifically by proteins containing tandem binding motifs that recognize SUMO and the target protein itself, like in the case of Srs2 recognition of sumoylated PCNA (10). Enhanced protein-protein interactions by sumoylation can occur between two proteins, as in Srs2 and PCNA, or alternatively, can result in the formation of large multimeric protein complexes. The best-characterized example of large protein complex formation by SUMO occurs through the sumoylation of the promyelocytic leukemia protein (PML). The combination of SUMO modification and SUMO binding activity of PML allows for large structures of PML to form PML-nuclear bodies that act as hubs for organizing and recruiting proteins containing SIMs (11,12).

Because sumoylation acts as a PTM, it affects the function of many key cellular processes including the stress response, chromatin structure, DNA repair and mitotic progression (13,14). The effects of sumoylation on each individual substrate protein can vary dramatically. Sumoylation can affect the enzymatic activity of a protein like thymine DNA glycosylase (TDG), where sumoylation promotes TDG enzymatic turnover (15). In addition, sumoylation can affect the stability of a target protein in two ways. Sumoylation can promote the degradation of proteins by recruiting SUMO-targeted ubiquitin ligases like RNF4, which ultimately targets that protein for degradation by the proteosome (6). In contrast, sumoylation of lysine residues can compete for ubiquitination of these resides to antagonize the degradation of a protein. Finally, the most common effect of sumoylation is the alteration of protein-protein interactions,

which can impact the localization, binding partners, and ultimately the function of the modified protein.

SUMO Conjugation and Deconjugation

Sumoylation occurs through a three enzyme cascade analogous to, but distinct from, the ubiquitination pathway (Figure 2). First, SUMO is processed into its mature form by SUMO-specific isopeptidases (16,17). After a C-terminal diglycine motif has been exposed, the heterodimeric SUMO-activating enzyme called Aos1/Uba2 activates SUMO (1). The E1 enzyme utilizes ATP to form a thiol-ester bond between SUMO and a cysteine residue on the E1 (1). Then, the E1 can interact with E2, the SUMO-conjugating enzyme called Ubc9, and transfer SUMO to a high-energy thiolester bond on Ubc9 (18,19). The SUMO is then transferred to the target protein, with or without the requirement of a SUMO E3 ligase.

Ubc9 can interact directly with target proteins and transfer SUMO to the ε-amino group of the substrate lysine. This can occur by Ubc9 recognizing the sumoylation consensus site, ψ −K-X-D/E, where ψ is a hydrophobic residue and X is any amino acid, and thus, and modify the K residue within this sequence. (20-23). In addition, Ubc9 can recognize target proteins through their SIM domains. The SUMO-charged Ubc9 is recognized by a SIM in the target protein, which facilitates Ubc9 interaction with the target protein and ultimately, sumoylation of a lysine residue on that protein (24). Notably, this second mechanism of Ubc9 transfer also explains the paralog specificity of modification observed in proteins like BLM, where the BLM SIM preferentially recognizes SUMO-2, resulting in its preferential modification by SUMO-2 (24).

In addition to direct SUMO modification by Ubc9, sumoylation can also require the activity of a SUMO E3 ligase. *In vitro,* the requirement for E3 ligases in SUMO conjugation assays depends on the concentration of Ubc9 (25). Thus, in the context of a cell, SUMO E3 ligases are likely to be critical for sumoylation. To date, multiple SUMO E3 ligases have been identified. The best-characterized family includes Siz1, Siz2, and Mms21 in yeast and the protein inhibitor of STAT (PIAS) proteins in higher eukaryotes, which all contain a conserved SP-RING domain. These RING-domain containing SUMO E3 ligases function as a scaffold to bring SUMO-charged Ubc9 into close proximity with the substrate protein in an analogous fashion to the ubiquitin RING E3 ligases. However, other non-RING E3 ligases have also been described, such as RanBP2 and Pc2, whose mechanisms for enhancing sumoylation of substrates are still not fully understood (26-29). Because there is only one SUMO E1 enzyme and one E2 enzyme, the E3 ligases are likely to be important for regulating substrate recognition and specificity of sumoylation *in vivo*.

In addition to regulation at the conjugation level, sumoylation can be regulated by deconjugation as well. The first SUMO isopeptidases identified were Ulp1 and Ulp2 in yeast (16,30). Since this time, six related isopeptidases have been identified in vertebrates (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7) (17). The SENPs belong to the CE class of cysteine proteases that contain a catalytic triad of aspartate, histidine and cysteine (31). These enzymes share a conserved C-terminal catalytic domain that confers unique paralog specificity for each isopeptidase (Figure 3A) (17). The SENPs also contain divergent N-terminal domains. The N-terminus of Ulp1 regulates localization and substrate specificity, and thus it has been suggested that the N-termini of the SENPs have

an analogous function (32). The vertebrate SENPs localize to unique cellular structures: SENP1 and SENP2 localize to NPCs, SENP3 and SENP5 to the nucleolus and SENP6 and SENP7 to the nucleoplasm (Figure 3B) (33-37). Notably, the isopeptidases are not functionally redundant, as the SENP1 and SENP2 knockout mice are embryonic lethal (38,39). More recently, other non-classical SUMO isopeptidases have been identified, including Wss1 in yeast and DeSI-1 and Uspl-1 in mammals (40-42). However, the functional significance of these isopeptidases remains to be determined.

Because sumoylation is reversible, it is the balance between SUMO conjugation and deconjugation that creates the steady state level of sumoylation in cells. This concept is demonstrated by the mechanism for SUMO-1 specific modification of RanGAP1. RanGAP1 is efficiently modified by SUMO-1 and SUMO-2/3 but only SUMO-1 modified RanGAP1 can bind RanBP2, which protects this modification from cleavage by isopeptidases. Thus, knockdown of either RanBP2 or SENP1 and SENP2 results in an increased accumulation of SUMO-2 modified RanGAP1, demonstrating the delicate balance between sumoylation and desumoylation (43). Notably, the interplay of dynamic conjugation and deconjugation results in a very low percentage of a given substrate to be sumoylated at a time, which has been termed the "SUMO enigma" (44). Precisely how such a small pool of a sumoylated substrate can have a profound effect on the function of a protein remains an important question in the field today.

SUMO is a regulator of mitosis

Sumoylation was shown to be critical for mitotic progression before SUMO was even discovered when Seufert and colleagues demonstrated that *ubc9* temperature

sensitive mutants arrest in G2/M (45). Shortly thereafter, the yeast form of SUMO, *smt3*, was identified as a high copy suppressor of *mif2* mutants, the vertebrate CENP-C homolog, suggesting a role for SUMO in centromere structure (46). Since these initial studies, genetic manipulation of the SUMO E1, E2, and E3 enzymes exhibited mitotic arrest phenotypes in *Drosophila melanogaster, Saccharomyces pombe, Danio rio*, and mammalian cells, demonstrating a conserved requirement for SUMO in mitotic progression (4,47-50). Surprisingly, dynamic desumoylation is also required for proper mitotic progression because knockdown or genetic manipulation of the SUMO deconjugating enzymes also leads to G2/M arrests (16,51-53). Thus, sumoylation and desumoylation are both required for mitotic progression, suggesting a critical temporal regulation for sumoylation throughout mitosis.

Microscopy studies have demonstrated that sumoylation is regulated spatially and temporally throughout the mitotic cycle. In *Saccharomyces cerevisiae*, *smt3* localizes to mitotic chromosomes, but in mammalian cells, the different SUMO paralogs localize to distinct mitotic structures (54). SUMO-1 localizes to the mitotic spindle and moves to the spindle midzone in anaphase, while SUMO-2/3 colocalizes with centromeres in the early phases and associates with whole chromosomes after metaphase (4). Thus, the different SUMO paralogs are uniquely regulated spatially in mitosis and also exhibit temporal regulation at the metaphase to anaphase transition.

The mechanisms regulating the dynamics of sumoylation in mitosis are still poorly understood. Recent data demonstrated that two SUMO E3 ligases, PIASy, and PIAS3, are centromere associated in mitosis, suggesting a role in promoting centromeric SUMO-2/3 modification (55-57). Furthermore, another SUMO E3 ligase called RanBP2

localizes to kinetochores and mitotic spindles, indicating a potential role in regulating SUMO-1 modification on the spindle (58,59). However, further studies are required to determine the functional impact of these E3 ligases on mitotic sumoylation. Notably, the yeast isopeptidase Ulp1 and the mammalian SENP5 also change localization in mitosis, indicating that these enzymes could also be regulating sumoylation dynamically in mitosis (51,60). Ulp1 is released from NPCs in mitosis to regulate septin sumoylation at the budneck and thus, is a genuine regulator of mitotic sumoylation (60). Thus, there is preliminary evidence that the SUMO E3 ligases and isopeptidases are regulating sumoylation temporally and spatially in mitosis.

As reviewed below, a number of hallmark studies have provided evidence that sumoylation is required at nearly every regulated step in mitosis. In the remainder of this chapter, I will highlight the roles of sumoylation in many of the key regulatory steps of mitosis including DNA condensation and decatenation, sister chromatid cohesion, chromosome segregation, and finally cytokinesis.

Chromosome Condensation

Sumoylation has been implicated as a regulator of chromosome condensation. One of the non-core subunits of the condensin complex, Ycs4 (the homolog of vertebrate CAP-D2), is sumoylated, but the function of this sumoylation is still unknown (61). However, there is evidence that sumoylation promotes chromosome condensation in flies and mammals. Hypocondensation of mitotic DNA occurs in mouse embryonic fibroblasts (MEFs) hypomorphic for Ubc9 and in flies containing a mutant form of the E3 ligase Su(var)2-10. (62,63). Because defects in chromosome condensation can be caused by

defects in general chromatin structure, the condensin complex or in DNA decatenation (64), further studies are required to determine which process sumoylation is regulating to produce these hypocondensation phenotypes.

DNA decatenation

Sumoylation also plays a pivotal role in the proper decatenation of DNA. For example, MEFs hypomorphic for Ubc9 have increased chromosome bridges and broken chromosomes, suggesting the DNA was catenated at the time of division (63). In *Xenopus laevis* extracts, topoisomerase IIα, the major decatenase enzyme, is preferentially modified by SUMO-2/3 and this modification has been proposed to occur specifically during mitosis (65). Even though sumoylation has no direct effect on the decatenase activity of topoisomerase IIα *in vitro* (65), evidence in multiple organisms suggests that SUMO regulates topoisomerase IIα targeting to centromeres. In yeast, expression of a topoisomerase IIα-SUMO fusion protein caused enrichment of topoisomerase IIα at centromeres (66). Furthermore, MEFs hypomorphic for the SUMO E3 ligase RanBP2 have reduced topoisomerase IIα recruitment to centromeres and exhibit high levels of chromosome bridges in anaphase, suggesting a defect in decatenation (67). A comparable phenotype is seen in HeLa cells with knockdown of another SUMO E3 ligase PIASγ, which has reduced recruitment of topoisomerase IIα to centromeres and heavily catenated DNA that cannot segregate (49). Thus, sumoylation of topoisomerase IIα at least partially regulates its activity through the proper targeting and recruitment to centromeres, which are sites of still heavily catenated DNA in mitosis.

However, why sumoylation acts as a signal for topoisomerase IIα recruitment to centromeres is still not understood. Treatment of HeLa cells with ICRF-187, a topoisomerase inhibitor, increases the sumoylation of topoisomerase IIα and results in the retention of SUMO-2/3 associated with mitotic chromosomes (68). Agostinho and colleagues interpreted this result to signify that sumoylation occurs on catalytically committed topoisomerase species, which were artificially stabilized by the inhibitor treatment (68). One prediction based on this model could be that sumoylation facilitates the recycling of topoisomerase IIα from DNA after it has been catalytically active. Notably, *in vitro* analyses have demonstrated that SUMO-modified topoisomerase IIα has a lower affinity for DNA than the unmodified forms, supporting a model for sumoylation facilitating the recycling and optimal activity of topoisomerase IIα (65). However, this recycling model for SUMO regulation of topoisomerase IIα does not explain why loss of sumoylation results in the failed recruitment of topoisomerase IIα to centromeres. Thus, further biochemical studies are required to determine precisely how sumoylation regulates topoisomerase II function temporally and spatially in mitosis.

Cohesion Maintenance

Sumoylation was first identified as a negative regulator of cohesion through genetic studies. In *S. cerevisiae*, either overexpression of the isopeptidase Ulp2, or knockdown of Ubc9 increased cohesion between sister chromatids, suggesting that sumoylation has a pro-separation function (69). This anti-cohesion activity of SUMO is at least partially explained by sumoylation of Pds5, a protein known to regulate sister chromatid cohesion. Pds5 mutants are defective in cohesion but their phenotypes are

suppressed by Ulp2 overexpression, suggesting that removal of SUMO from Pds5 promotes the maintenance of cohesion (70). Furthermore, phosphorylation of Ulp2 by the kinase cdc25 inactivates the pro-cohesion activity of Ulp2, providing an explanation for the temporal regulation of this process (71). This mechanism has yet to be demonstrated in vertebrates. Furthermore, a recent study in DT40 chicken cells demonstrated that SENP1$^{-/-}$ cells, which are in a hyper-sumoylation state, have reduced cohesive capacity in the presence of microtubule destabilizing agents, supporting the hypothesis that sumoylation is a negative regulator of cohesion in vertebrates (72).

In addition to Pds5, multiple components of the cohesion complex itself have been shown to be sumoylated, including Scc1 (73-75). One of the greatest challenges in studying the function of sumoylated complexes like cohesin is the production of SUMO-deficient mutants for all complex subunits. For example, a lysine-less Scc1 mutant has normal sister chromatid cohesion in mitosis but it is unclear if sumoylation of other subunits compensates for SUMO-less Scc1 (74). Thus, to evaluate the functional role of a completely SUMO-deficient cohesin complex, Almedawar and colleagues created an Scc1 fused to the SUMO isopeptidase Ulp1 catalytic domain. *S. cerevisiae* cells expressing the Scc1-Ulp1 catalytic domain fusion were not functional for maintaining centromere cohesion in metaphase (75). Surprisingly, this result suggests that sumoylation is a positive regulator for cohesion, which opposes the function of SUMO in the Pds5 pathway. Notably, MEFs hypomorphic for Ubc9 also exhibited reduced cohesion, demonstrating that sumoylation can also be a pro-cohesion signal in vertebrates (63). Thus, cohesion is an example of one of the many cellular processes that is regulated by sumoylation in a dichotomous manner. Sumoylation of different proteins in the same

pathway, like Pds5 and Scc1, can have opposing effects, highlighting the importance of studying how sumoylation affects cellular functions at the level of the substrate protein.

Chromosome segregation

Genetic studies have implicated sumoylation and desumoylation as critical regulators of chromosome segregation. For example, SUMO E3 ligase mutants in *S. cerevisiae* and *S. pombe* exhibit increased mini-chromosome loss (66,76). Furthermore, endogenous chromosome segregation defects have been observed in flies and mammalian cells with mutants in Su(var)2-10 and RanBP2, supporting a requirement for sumoylation in chromosome segregation (62,77). These defects in chromosome segregation observed in a hypo-sumoylation environment are at least partially due to faulty chromosome alignment because chromosome congression defects are observed with overexpression of SENP2 or knockdown of Ubc9 (4). Notably, desumoylation is also required for proper chromosome segregation, as siRNA knockdown of SENP6 also results in the misalignment of chromosomes (53). Because chromosome segregation depends on proper kinetochore-microtubule interactions and the mitotic spindle, the functions of SUMO in each of these processes will be discussed next.

Centromere and kinetochore structure

In mammalian cells, sumoylation is critical for regulating centromere structure by two mechanisms: maintaining the chromatin environment and regulating the stability of proteins in the constitutive centromere associated network (CCAN). The human centromere is composed of α-satellite DNA that extends up to 4 Mbp and is a

predominantly heterochromatin environment (78). Sumoylation of the heterochromatin protein, HP1α, is critical for its targeting to the centromere and thus, establishing the heterochromatin environment of the centromere. Notably, this sumoylation must be a transient event, as SENP7 desumoylates HP1α after recruitment to allow proper HP1α enrichment at the centromere (52,79). Thus, HP1α is a nice example of the "SUMO enigma," where low levels of sumoylation are critical for function (44). In addition, SUMO regulates the stability of two of the proteins present in the CCAN called CENP-H and CENP-I. When sumoylated, CENP-H and CENP-1 are targeted for degradation by the proteosome via the SUMO-targeted ubiquitin E3 ligase called RNF4 (53). However, CENP-H/I protein levels are kept stable by the activity of the chain editing isopeptidase SENP6, suggesting a role for sumoylation in maintaining the proper complement of proteins required for the CCAN (53). Thus, sumoylation appears to be critical for the basic structure of the centromere, affecting the chromatin structure and CCAN proteins.

Notably, sumoylation has also been implicated as a regulator of the outer kinetochore, which is layered on top of the CCAN and regulates kinetochore and microtubule attachments. Knockdown of the SUMO E3 ligase RanBP2 results in the failed recruitment of kinetochore proteins (CENP-E, dynein, and CENP-F) and spindle assembly checkpoint proteins (Mad1, Mad2, and Zw10), suggesting that sumoylation facilitates the formation of protein complexes at kinetochores (80). A role for SUMO in the formation of dynamic subcomplexes in the kinetochore was confirmed when CENP-E targeting to kinetochores was shown to be SIM-dependent (4). However, which kinetochore proteins are being sumoylated to promote this complex formation is still poorly understood. Besides protein complex formation, the function of sumoylated

kinetochore proteins is still largely unknown (Table 1). However, it is clear that two components of the RZZ complex, Rod and ZW10, are important for recruiting the SUMO E3 ligase PIASy to kinetochores, which is required for strong SUMO-2/3 association with kinetochores in *X. laevis* (55). PIAS3 and RanBP2 are also found on kinetochores, so it will be important determine how these E3 ligases regulate the sumoylation of kinetochore proteins and thus, the formation of dynamic SUMO-dependent protein complexes in the kinetochore (57-59).

Mitotic spindle

Sumoylation is important for proper spindle structure and positioning in the cell. In *S. cerevisiae,* a mutant allele of *smt3* was identified to have short mitotic spindles, resulting in a reduced distance between sister chromatids after separation (54). Furthermore, *smt3* and *ubc9* mutants also have a spindle alignment defect that prevents proper asymmetrical division at the budneck, which was at least partially mediated by sumoylation of Kar9 (81). These phenotypes have yet to be characterized in vertebrates, but RanBP2 localization to mitotic spindles suggests a conserved role for sumoylation in spindle function (59). Notably, desumoylation also regulates spindle function. In mammalian cells, SENP6 knockdown results in an increased distance between spindle poles and between sister chromatid kinetochores, suggesting a defect in regulation of spindle tension (53). Thus, there is preliminary evidence in the literature that SUMO is regulating spindle dynamics but further investigation is required to understand the molecular mechanisms involved.

Cytokinesis and septin sumoylation

Finally, a role for sumoylation in cytokinesis has been suggested by studies of sumoylated septins in yeast (23,60). Yeast strains lacking septin sumoylation can cycle normally, but accumulate old bud scars because sumoylation is required for the proper disassembly of septin rings after mitotic exit (23). Siz1 and Ulp1 temporally regulate septin sumoylation, providing another example for the requirement for dynamic sumoylation in regulating mitotic processes (60,82). Septin sumoylation has not been as well characterized in vertebrates, but there are examples of cytokinesis defects in mammalian cells upon manipulation of the SUMO pathway. For example, knockdown of RanBP2 or expression of a SUMO-less Aurora B results in an increase in multinucleated cells (80,83). Dynamic sumoylation also appears to be critical for cytokinesis in vertebrates, as knockdown of SUMO isopeptidases also results in an increase in multinucleated cells (84). Future studies are required determine precisely how sumoylation affects proper cytokinesis divisions.

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SUMO is a master regulator of mitosis

In this thesis, I will investigate the functional role of sumoylation in mitosis through a two-pronged approach. Because sumoylation can affect mitotic processes in a dichotomous fashion, as was highlighted throughout this chapter, it is critical that more mitotic SUMO-modified proteins be identified. Thus, in Chapter 2, I utilized a combination of cell fractionation, immunopurification, and mass spectrometry to identify the SUMO-2/3 modified proteins associated with mitotic chromosomes. In addition, I conducted an analysis of two SUMO isopeptidases, SENP1 and SENP2, to better

understand the temporal and spatial regulation of sumoylation in mitosis, which is presented in Chapter 3. For this study, I utilized a combination of overexpression and knockdown studies to decipher the functional role of SENP1 and SENP2 in mitotic progression. In Chapter 4, I conducted a literature analysis of the role of sumoylation in chromatin structure because understanding how sumoylation impacts chromatin structure could have important implications on its mitotic functions in centromere structure and chromosome condensation. Finally, I conducted a more targeted study of SENP1 to further characterize the molecular mechanism involved in a metaphase to anaphase progression defect observed with SENP1 knockdown, which is presented in the Appendix. In its entirety, this thesis provides a foundation for further analysis of the roles of sumoylation in regulating progression through mitosis, and for understanding how SUMO isopeptidases contribute to this regulation.

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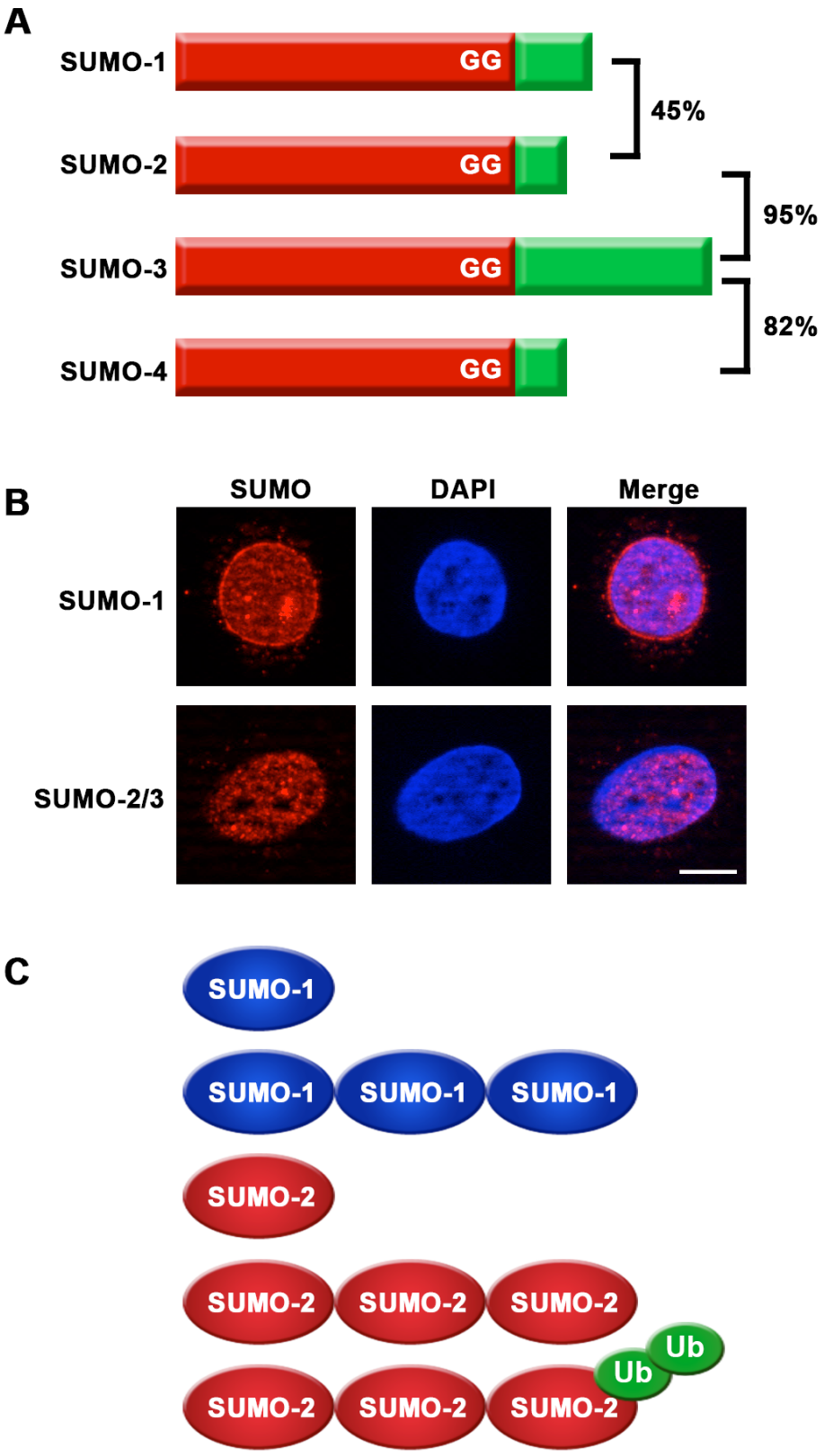
Figure 1-1. The SUMO paralogs modify distinct proteins and act as unique signals. **(A)** Cartoon schematic showing the precursor forms of the four SUMO paralogs found in vertebrates. The diglycine motif (GG) that must be exposed by isopeptidase cleavage to form mature SUMO is shown. Percent identity between the SUMO paralogs is also depicted. **(B)** HeLa cells were fixed, permeabilized and stained with antibodies specific for SUMO-1 or SUMO-2/3. DNA was stained with DAPI. SUMO-1 localizes diffusely in the nucleoplasm, to the nuclear rim, and is observed in bright nuclear foci that represent PML nuclear bodies. SUMO-2/3 localizes diffusely in the nucleoplasm and is also observed in PML nuclear bodies but is excluded from nucleoli and the nuclear rim. Bar = 10 μm. **(C)** Cartoon schematic showing the many unique signals that are created by sumoylation.


A
SUMO-1
GG
45%
SUMO-2
GG
95%
SUMO-3
GG
82%
SUMO-4
GG
B
SUMO
DAPI
Merge
SUMO-1
SUMO-2/3
C
SUMO-1
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SUMO-2

Figure 1-2. The SUMO modification pathway. SUMO is translated as a precursor protein that is cleaved to mature SUMO by a family of isopeptidases (SENPs). Mature SUMO is activated by the E1 enzyme, Uba2/Aos1, and transferred to the SUMO conjugating enzyme Ubc9. Ubc9 interacts with the SUMO E3 ligases to directly transfer SUMO to a substrate protein. Desumoylation of a substrate occurs through the activity of the SENPs.

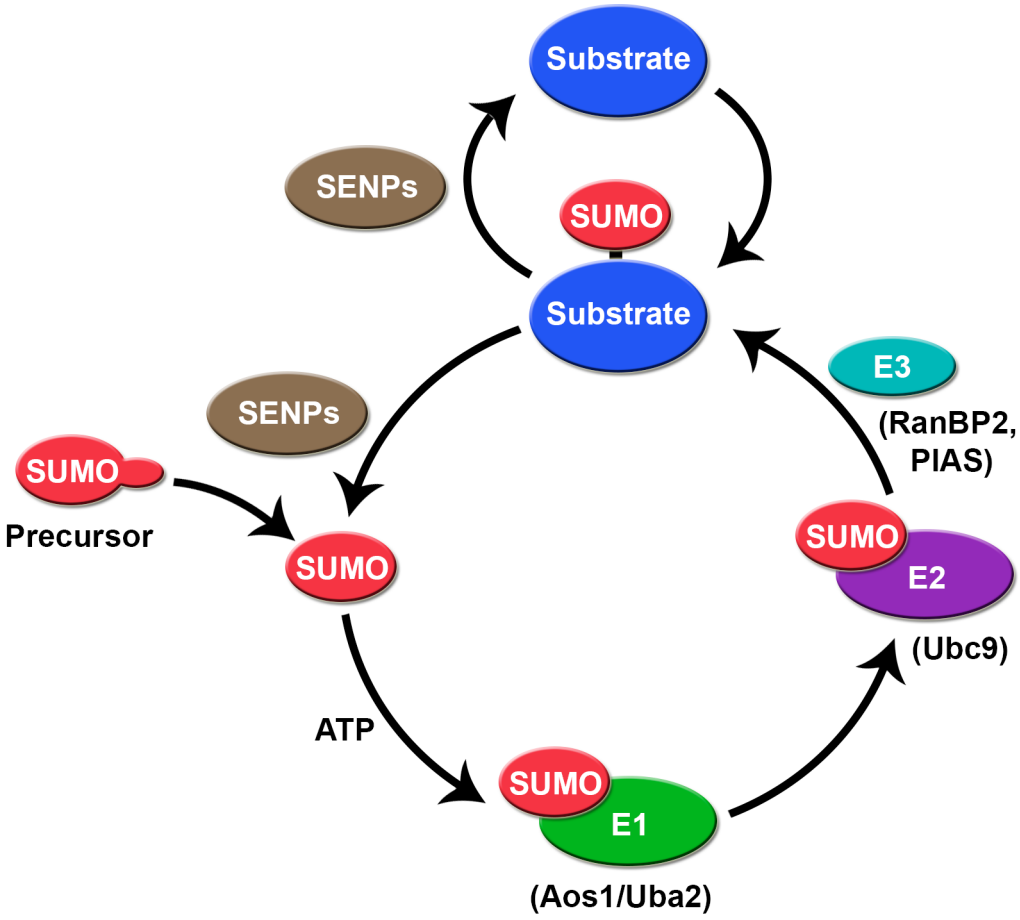
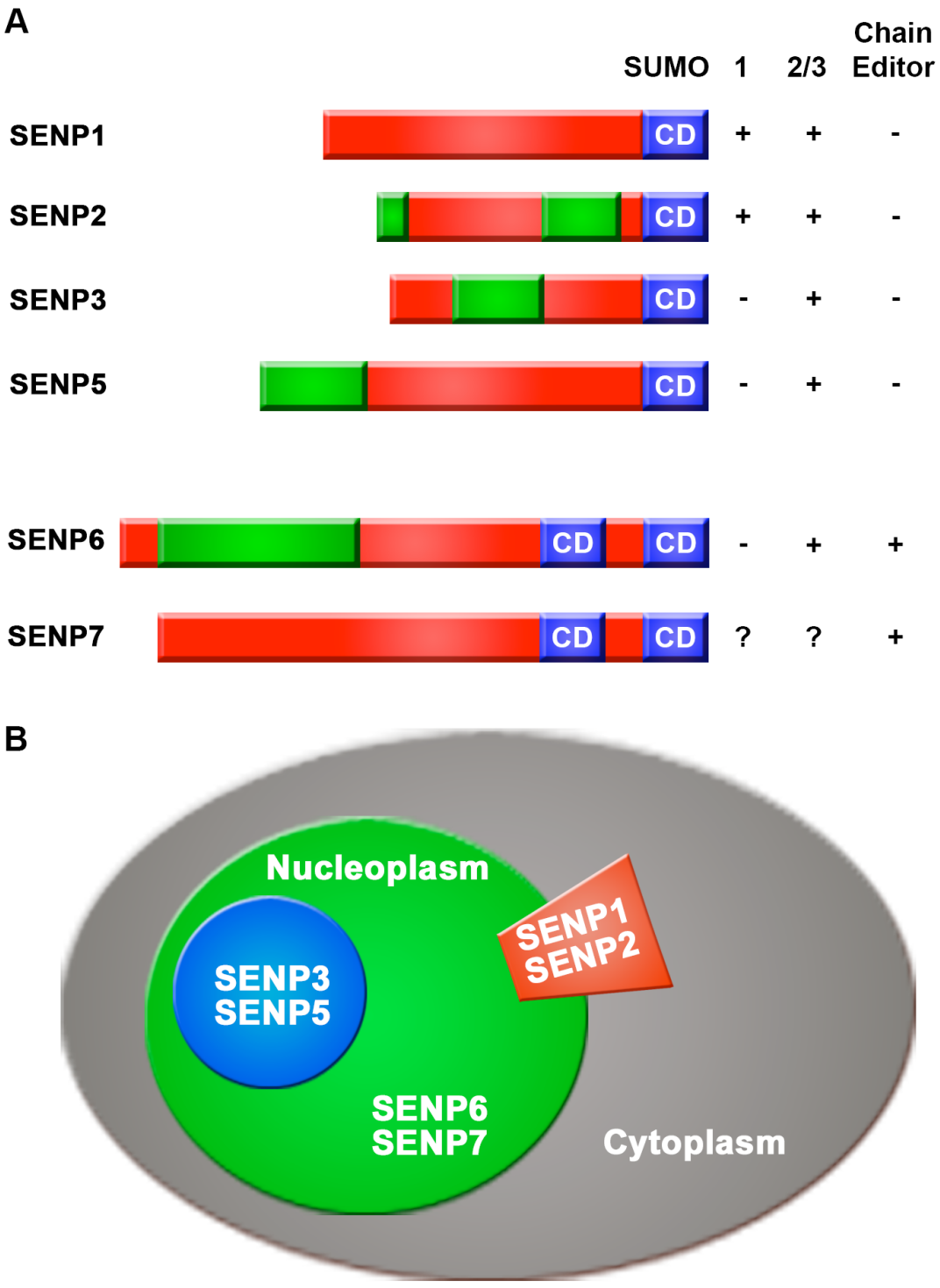

Substrate
SENPs
SUMO
Substrate
E3
(RanBP2,
PIAS)
SENPs
SUMO
Precursor
SUMO
SUMO
E2
(Ubc9)
ATP
SUMO
E1
(Aos1/Uba2)

Figure 1-3. The domain structure and localization of the SENPs, a family of SUMO isopeptidases. (A) Cartoon schematic showing the domain structure of the isopeptidases. The conserved catalytic domains are shown in blue (CD) and the divergent N-termini are shown in red. Known targeting signals within the N-terminus are highlighted in green. Differences in the isopeptidase activity inherent in the catalytic domains of the SENPs are summarized on the right (36). **(B)** Cartoon schematic showing the unique cellular localizations of the SENPs in interphase.


A
SUMO
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SENP1
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SENP6
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+
SENP7
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Nucleoplasm
SENP3
SENP5
SENP1
SENP2
SENP6
SENP7
Cytoplasm

Table 1-1. Sumoylated proteins in mitosis. This table lists the previously identified sumoylated proteins in mitosis. The E3 ligases and isopeptidases known to interact with these proteins are also listed. However, many of the studies did not conduct exhaustive analysis of the E3 ligases or isopeptidases, so other sumoylation enzymes may also interact with each substrate.

Protein	Mitotic Function	E3 ligase	Isopeptidase	Function of sumoylation	References
Aurora B	Chromosome passenger complex	PIAS3	SENP2	Defective chromosome segregation and cytokinesis	(57,83)
Bir1 (Survivin)	Chromosome passenger complex			Unknown; sumoylation depends on microtubule attachment	(87)
Borealin	Chromosome passenger complex	RanBP2	SENP3	Unknown	(77)
BubR1	Spindle assembly checkpoint		SENP1 SENP2	Unknown	(4,85)
CENP-C	Centromere structure			SUMO overexpression suppressed CENP-C mutants	(46,86)
CENP-E	Chromosome congression at metaphase plate		SENP2	SUMO binding is required for kinetochore recruitment	(4)
CENP-H/I	Centromere structure		SENP6	Sumoylation targets CENP-I/H for degradation	(53)
Cep3	Kinetochore			Unknown; sumoylation depends on microtubule attachment	(87)
Ndc10	Kinetochore	Siz1 Nfi1	Ulp2	Unknown; sumoylation depends on microtubule attachment	(87)

Protein	Mitotic Function	E3 ligase	Isopeptidase	Function of sumoylation	References
Ndc80	Kinetochore-microtubule attachment			Unknown	(87)
Nuf2	Kinetochore		SENP2	Unknown	(4)
PARP1	Unknown	PIASy		Unknown	(25)
Pds5	Cohesion	Nfi1	Ulp2	Sumoylation promotes cohesion dissolution	(70,71)
RanGAP1	Unknown; Localizes to mitotic spindles and kinetochores	RanBP2	SENP1 SENP2	Forms complex with RanBP2, SUMO-RanGAP1, and Ubc9	(43,58,59)
Scc1 (Cohesin complex)	Cohesion	Mms21	Ulp1 (catalytic domain)	Sumoylation of cohesin complex is required for sister chromatid cohesion	(73-75)
Septins	Cytokinesis	Siz1	Ulp1	Sumoylation required for septin ring disassembly	(23,60,82)
Topoisomerase II	DNA decatenation	PIASy RanBP2 Siz1/2 PIASγ		Sumoylation regulates its centromeric localization	(49,65-67)
Ycs4 / CAPD2	Chromosome condensation			Unknown	(61)

CHAPTER 2

IDENTIFICATION OF SUMO-2/3 MODIFIED PROTEINS ASSOCIATED WITH MITOTIC CHROMOSOMES

ABSTRACT

SUMO modification of proteins (sumoylation) is essential for mitotic progression from yeast to humans, but only a limited number of sumoylated proteins with functions in mitosis have been discovered. Vertebrates express three SUMO paralogs, SUMO-1, SUMO-2 and SUMO-3, which are uniquely associated with chromosomes in mitosis. In this study, we used chromosome spreads to more precisely define the localization of endogenous SUMO-2 and SUMO-3 to the centromere as well as the chromosome protein scaffold. Furthermore, we developed methodologies for the immunopurification of endogenous SUMO-2 and SUMO-3 modified proteins from cell extracts. Using nLC-ESI-MS/MS, we identified proteins immunopurified from mitotic chromosome fractions and from G_0 nuclei as a comparison. We identified a total of 244 putative sumoylated proteins (results have been deposited in the ProteomeXchange, identifier PXD000381), with 149 of these proteins being associated with the mitotic chromosome fraction. We identified proteins with known mitotic functions and that localize to centromeres, kinetochores and the chromosome protein scaffold, consistent with SUMO-2 and SUMO-3 localization. Our results provide a foundation for characterizing the functions of sumoylation in regulating diverse aspects of mitotic function from chromosome segregation to cytokinesis.

INTRODUCTION

The small ubiquitin-related modifiers (SUMOs) are covalently conjugated to other proteins and regulate many essential cellular processes including transcription, DNA repair, and mitosis (1,2). Invertebrates express only one SUMO to regulate these diverse processes but vertebrates express three SUMO paralogs (SUMO-1, SUMO-2, and SUMO-3). While SUMO-2 and SUMO-3 share 97% identity and are referred to collectively as SUMO-2/3, SUMO-1 only shares ~50% identity with SUMO-2/3 (3). All three paralogs are conjugated to target proteins through an enzymatic cascade involving an E1 activating enzyme, an E2 conjugating enzyme and a family of SUMO E3 ligases. (3). Once sumoylated, the modified protein is often recognized by downstream factors containing SUMO-interacting motifs (SIMs), which can affect protein complex formation, localization, and/or stability (3). Paralog-selective SIMs may underlie the distinct effects of the different SUMO paralogs (4-6). In general, however, paralog-specific functions are still poorly understood. SUMO-modified proteins are also recognized by a family of cysteine isopeptidases (SENPs) that deconjugate SUMO, reversing the sumoylation process (7). As a relatively small fraction of most sumoylated proteins is modified at steady state, dynamic sumoylation appears to underlie many of the diverse cellular functions of the SUMO pathway (8).

Like phosphorylation and ubiquitylation, sumoylation is increasingly being recognized as an important mitotic regulator. Early genetic studies in yeast discovered an important role for sumoylation in mitotic progression (9,10). Subsequent studies from yeast to humans further demonstrated that sumoylation and desumoylation are both critical for mitotic progression, chromosome condensation, and sister chromatid

segregation (11). In mammalian cells, sumoylation is globally down-regulated as cells enter mitosis, however SUMO-1 and SUMO-2/3 are uniquely regulated following mitotic entry. SUMO-1 modified proteins localize predominantly to the mitotic spindle and modification by SUMO-1 remains low as mitosis progresses. In contrast, SUMO-2/3 modified proteins localize to mitotic chromosomes and modification by SUMO-2/3 increases from metaphase to telophase. This increase in SUMO-2/3 modification correlates with immunofluorescence data showing SUMO-2/3 restricted to the centromere/kinetochore in early mitosis, but along the length of the chromosomes in the later stages (5). Understanding the functional significance of the temporal and spatial changes in SUMO-2/3 modification during mitotic progression requires the identification of relevant SUMO-modified proteins. However, a comprehensive analysis of proteins modified by SUMO specifically during mitosis has not been previously reported.

To date, the best-characterized functions for sumoylation in mitosis have come from targeted analyses of a limited number of SUMO-modified proteins. For example, sumoylation of topoisomerase IIα has been shown to be critical for the proper decatenation of sister chromatids at the metaphase to anaphase transition (12-16). In addition, sumoylation of centromere and kinetochore proteins have been shown to be critical for kinetochore assembly and function (5,7,17-19). However, sumoylation outside of the kinetochores and centromeres is largely still unexplored.

To develop a more complete understanding of the roles of sumoylation in mitosis, we have analyzed SUMO-2/3 modification of proteins on mitotic chromosomes at a global level. We refined the localization of SUMO-2/3 modified proteins in prometaphase arrested cells, showing that SUMO-2/3 localizes to the centromere and the

chromosome protein scaffold. Furthermore, we developed a procedure for immunopurifying sumoylated proteins using an antibody recognizing endogenous SUMO-2/3. Using this procedure in combination with mass spectrometry, we identified SUMO-2/3 modified proteins associated with purified mitotic chromosomes and, for comparison, G_0 nuclei. We identified a total of 244 putative SUMO-2/3 modified proteins, with 149 of these proteins being identified in the mitotic chromosome fraction.

MATERIALS AND METHODS

Cell culture and synchronization

For immunofluorescence, HeLa cells were cultured on glass coverslips at 37°C and 5% CO_2 in Dulbecco's modified eagle medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 10 mM HEPES (pH=7.2-7.5). For SUMO-2/3 immunopurifications, HeLa cells were grown in suspension cultures at 37°C and 5% CO_2 in minimum essential medium eagle (Sigma) supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin, and 2 g/L sodium bicarbonate. HeLa cells were synchronized with an overnight treatment of 100 ng/ml nocodazole prior to harvest. This resulted in an approximately 50% mitotic population, defined by counting condensed propidium iodide-stained DNA by fluorescence microscopy.

Antibodies

The monoclonal antibody to SUMO-2/3 (8A2) (5) was purified from mouse ascites fluid by DEAE ion exchange chromatography (20) and immobilized onto Affigel-10 beads (BioRad) according to the manufacturer's protocol. For each immunopurification experiment, 6.5 mg of 8A2 (experimental) or 6.5 mg of mouse control IgG (Protein Mods LLC, Wisconsin) were immobilized onto 2 ml of Affigel-10 beads. The other antibodies used in this study were obtained from the following sources: CREST human auto-antibodies from Dr. Ted Salmon (University of North Carolina, NC); anti-TIF1b (ADI-KAM-TF200) from Enzo Life Sciences; anti-topoisomerase IIa (sc-13058) from Santa Cruz Biotechnology; anti-SMC4 from Dr. Tatsuya Hirano (Riken, Japan); anti-phospho-histone H3-Ser10 (06-570) from Upstate-Millipore); anti-histone 3 (39163) from Active

Motif; anti-Hsp90 (610418) from BD Transduction Laboratories; and anti-KIF4A from Genetex (GTX115579).

Immunofluorescence microscopy

To obtain chromosome spreads, HeLa cells were treated with 100 ng/ml nocodazole for 4 hours, permeabilized, fixed, and post-permeabilized as previously described (21). Permeabilization buffers were supplemented with 20 mM N-ethylmaleimide (NEM) to inhibit SUMO-specific isopeptidases. Immunostaining was done as described previously (22). Images were collected using a Zeiss Observer.Z1 fluorescence microscope with an Apotome VH optical sectioning grid and were processed using the AxioVision Software Release 4.8.2.

Cell fractionation

For mitotic samples, four liters of synchronized HeLa cells were harvested by centrifugation and a chromosome fractionation was conducted using a modification of described protocols (23). Notably, the lysis buffer was supplemented with 10 mM NEM. After dounce lysis, lysates were spun at 200 x g for 5 minutes to remove unlysed cells and intact nuclei. The lysate was layered onto 15% sucrose cushion and spun for 30 minutes at 2,000 x g. The supernatant was removed and discarded. The pellet was resuspended in RIPA Buffer (20 mM Tris-HCl pH=7.5, 150 mM sodium chloride, 2mM EDTA, 1% sodium deoxycholate, 1% triton x-100) supplemented with 1% SDS, 10 mM NEM and protease inhibitors (5 mg/ml leupeptin, 5 mg/ml pepstatin A, and 1 mM PMSF) and used as the soluble chromosome fraction in subsequent steps.

For G_0 samples, the initial lysis steps were identical to the chromosome fractionation protocol. After dounce lysis, lysates were spun at 200 x g for 5 minutes to pellet intact nuclei and the soluble fraction was removed. The nuclei pellets were resuspended in RIPA Buffer supplemented with 1% SDS, 10 mM NEM and protease inhibitors and used as the soluble nuclear fraction in subsequent steps.

Immunopurification

The chromosome fraction or nuclear fraction was sonicated (3 times for 15 sec) and diluted 1:10 in RIPA supplemented with 10 mM NEM and protease inhibitors to reduce the SDS concentration to 0.1%. The sample was spun for 2 hours at 50,000 x g. The supernatant was collected, passed through a 0.22 mm filter. The input was split equally between Affigel-10 beads containing immobilized SUMO-2/3 antibody or mouse IgG control antibody 4˚C (see antibody section for more information). Samples were washed 4X with RIPA buffer supplemented with 0.1% SDS and 4X with the elution buffer (20 mM Tris-HCl pH = 7.5, 500 mM sodium chloride, 2 mM EDTA, 0.1% sodium deoxycholate, 0.1% triton x-100 and 0.1% SDS). Samples were eluted in elution buffer supplemented with 0.5 mg/ml of an 8A2 epitope-specific peptide (IRFRFDGQPINE), TCA precipitated, and analyzed by mass spectrometry. For validation of the mass spectrometry results, immunopurification elutions were analyzed by SDS-PAGE and immunoblotting using protein-specific antibodies.

Mass spectrometric analysis

Samples were resuspended in 30 ml XT sample buffer and separated via 4-12% Bis-Tris Criterion XT SDS-PAGE with XT-MES buffer (BioRad). Gels were stained with Coomassie brilliant blue, and each lane was cut out as a single section. Each gel section was sliced into 3 mm^2 pieces, and subjected to three cycles of dehydration (acetonitrile 20 min) and rehydration (deionized water 20 min). Following a final dehydration step (20 min SpeedVac), gel pieces were rehydrated in 50 mM ammonium bicarbonate pH 8.3 containing 1.5 mg TPCK trypsin (Promega), and incubated at 37°C for 16 hr. Peptides were extracted from the gel pieces using three dehydration/rehydration steps (as above). Eluted peptides were pooled, lyophilized and resuspended in 5% (v/v) acetonitrile in 0.1% (v/v) formic acid for MS analysis.

Analytical columns (75 μm inner diameter) and pre-columns (100 μm) for LC-MS analysis were prepared in-house from silica capillary tubing (InnovaQuartz, Phoenix, AZ), and packed with 3 μm 100Å C18-coated silica particles (Michrom). Analytical columns were fitted with metal emitters (Thermo Proxeon) using zero dead volume connections. Peptides were subjected to nanoflow liquid chromatography - electrospray ionization - tandem mass spectrometry (nLC-ESI-MS/MS), using a 90 min reversed phase (10-40% acetonitrile, 0.1% formic acid) buffer gradient running at 250 nL/min on a Proxeon EASY-nLC pump in-line with a hybrid linear quadrupole ion trap (Velos LTQ) Orbitrap mass spectrometer (Thermo Fisher Scientific). A parent ion scan was performed in the Orbitrap, using a resolving power of 60,000. Simultaneously, up to the twenty most intense peaks were selected for MS/MS (minimum ion count of 1000 for activation) using standard CID fragmentation. Fragment ions were detected in the LTQ. Dynamic

exclusion was activated such that MS/MS of the same *m*/*z* (within a 10ppm window, exclusion list size 500) detected two times within 15 sec were excluded from analysis for 30 sec.

Thermo .raw files were uploaded to the ProHits (24) analytical suite and converted to .mzXML format using ReAdW software. Data were searched using X!Tandem (25) against human ORFs (RefSeq v45). Search parameters specified a parent MS tolerance of ±15ppm, and an MS/MS tolerance of 0.4 Da, with up to two missed cleavages for trypsin. Oxidation of methionine and tryptophan, ubiquitylation of lysine, and alkylation of cysteine (by NEM) were allowed as variable modifications. Statistical validation of the results was performed using Peptide Prophet and Protein Prophet (26,27) as part of the trans-proteomic pipeline. For each search, the Protein Prophet probability at a 1% false discovery rate and minimum of 2 unique peptides were used as cutoff values to generate SAINT-compatible input files. SAINT parameters were as follows: 5000 iterations, low mode Off (0), mincFold 1 and normalization off (0) (28). SAINT cutoff values 0.8 MAX SAINT, 0.6 Avg SAINT were used to generate a high-confidence list of protein identifications. The mass spectrometry proteomics data have been deposited to the ProteomieXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000381 (29).

RESULTS

SUMO-2/3 localization on mitotic chromosomes

We previously demonstrated that SUMO-2/3 localize to mitotic chromosomes throughout mitosis using fixed, intact mitotic cells (5). To more precisely investigate the localization of SUMO-2/3 on mitotic chromosomes, we analyzed HeLa cell chromosome spreads by immunofluorescence microscopy. One chromosome is depicted for clarity (Figure 2-1) but whole fields for each chromosome spread are also shown (Figure 2-2). Co-staining with human CREST auto-antibodies that recognize CENP-A, CENP-B, and CENP-C (30) (Figure 2-1A) shows that SUMO-2/3 localizes to the paired sister chromatid centromeres. These results are consistent with the co-localization of SUMO-2/3 with CENP-B that has been previously demonstrated in intact mitotic cells (5).

Intriguingly, SUMO-2/3 was also detected on the chromosome arms. To determine where SUMO-2/3 localizes more specifically, we conducted co-localization studies with antibodies to a histone marker, phospho-H3 (Figure 2-1B), and a chromosome scaffold marker, Smc4, a primary subunit of the condensin complex (Figure 2-1C). The SUMO-2/3 signal does not co-localize with the peripheral phospho-H3, demonstrating that it is not globally present throughout mitotic chromosomes. However, SUMO-2/3 partially overlaps with Smc4, indicating that SUMO-2/3 is present in the chromosome protein scaffold. These results are similar to a previous report showing SUMO-2/3 on the chromosome protein scaffold in cells treated with a topoisomerase inhibitor (31). Collectively, these results demonstrate that endogenous SUMO-2/3 localizes to both the centromere and the chromosome protein scaffold during mitosis.

Cell synchronization

To identify proteins modified by SUMO-2/3 in mitosis, and to characterize the modification of these proteins in a separate phase of the cell cycle, we synchronized HeLa cells in both mitosis and G_0. We isolated G_0 cells through serum starvation and mitotic cells through nocodazole synchronization and release. We obtained distinct cellular populations with only 2.5% (+/- 0.5%) of cells in mitosis in G_0 preparations and 53.2% (+/- 5.1%) of cells in mitosis in mitotic preparations (Figure 2-3). Our method for mitotic synchronization resulted in a predominantly prometaphase population of cells, but other phases of mitosis were also present to varying degrees between the two biologically independent preparations (Figure 2-3B). Even though the mitotic synchronization did not reach 100%, further enrichment was achieved during cellular fractionation when mitotic chromosomes were separated from intact interphase nuclei (see experimental approaches).

Cell fractionation isolation

Mitotic chromosomes from nocodazole treated cells, or intact nuclei from G_0 cells, were isolated by differential centrifugation and analyzed by SDS-PAGE. Coomassie Blue staining of whole cell lysates, isolated G_0 nuclei and mitotic chromosomes fractions revealed distinct protein profiles (Figure 2-4A). Furthermore, the fractionation successfully separated a well-characterized cytosolic protein, heat shock protein 90 (Hsp90), from nuclear histone 3 (H3) as revealed by immunoblot analysis (Figure 2-4B). Significantly, immunoblot analysis also revealed that unconjugated SUMO-2/3 remained in the soluble protein fractions, while high-molecular weight

SUMO-modified proteins were found in both the soluble and pellet fractions (Figure 2-4B). Thus, by performing cellular synchronization and fractionation prior to immunopurification, we enriched for high molecular weight SUMO-2/3 modified proteins associated with mitotic chromosomes or G_0 nuclei and removed unconjugated SUMO-2/3.

Immunopurification and identification of endogenous SUMO-2/3 modified proteins

To identify sumoylated proteins associated with isolated mitotic chromosomes and G_0 nuclei, we conducted SUMO-2/3 immunopurifications and analyzed the proteins by nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS) (Figure 2-5A). Chromosome or nuclear pellets were solubilized with SDS and proteins were immunopurified using the SUMO-2/3 specific monoclonal antibody, 8A2, conjugated to agarose beads. As a control, immunopurifications were also performed using mouse IgG similarly conjugated to agarose beads. Isolated proteins were eluted using an 8A2 epitope-specific peptide and analyzed by SDS-PAGE. Immunoblotting demonstrated that high molecular weight SUMO-2/3 modified proteins were specifically purified with the SUMO-2/3 antibody but not with the mouse IgG control (Figure 2-5B). Furthermore, silver stain analysis demonstrated that the SUMO-2/3 immunopurification uniquely enriched for high molecular weight proteins, relative to the IgG control (Figure 2-5C).

We conducted immunopurifications from two independently prepared fractions of purified mitotic chromosomes and G_0 nuclei. Purified proteins were separated by SDS-PAGE to remove the elution peptide and analyzed by nLC-ESI-MS/MS. The significance

analysis of interactome (SAINT) approach was used to identify proteins unique to the SUMO-2/3 immunopurifications (28). SAINT utilizes statistical analysis of the total number of spectra obtained for individual proteins in experimental and control immunopurifications to determine the probability that a protein is unique to the experimental sample. Using this method, we identified a total of 244 proteins specific to SUMO-2/3 immunopurifications from mitotic chromosomes and G_0 nuclei (Table 2-1). Of these proteins, 149 were identified from fractions of purified mitotic chromosomes and 184 were identified from G_0 nuclei. 89 of the identified proteins were common to both the mitotic chromosome fraction and G_0 nuclei. These results indicate that at least half of the sumoylated proteins associated with mitotic chromosomes are also SUMO modified in G_0. Proteins identified uniquely in mitosis or G_0 may be sumoylated in a cell cycle dependent manner, although further studies are required to determine if this is the case.

Bioinformatic analysis of identified proteins

We first compared the proteins identified in our study to three recent proteomics studies, two by Hay and colleagues in which >750 sumoylated proteins were identified from asynchronous cells after heat shock (1,32), and a third by Melchior and colleagues in which proteins modified by endogenous SUMO-2/3 were identified from whole cell lysates of asynchronous cells (33) (Figure 2-6). Overall, 63% of the proteins identified in our study were identified previously. Notably, 83% of the proteins we identified as sumoylated in both G_0 and mitosis were identified in the Hay and Melchior proteomic studies, suggesting that these proteins are likely high abundance SUMO substrates that

are detected under various cellular conditions. In contrast, only 51% and 55% of the proteins identified specifically in G_0 and mitosis, respectively, were identified previously. Thus, the cell synchronization and fractionation techniques utilized in this study may promote the detection of lower abundance SUMO substrates that are omitted from large scale whole cell lysate analyses.

We next compared the list of putative SUMO-2/3 substrates identified in our mitotic chromosome fraction to proteins identified in other mass spectrometry studies focused on chromosome composition. In particular, we compared our results to two proteomics studies by Earnshaw and colleagues that identified proteins on the non-histone protein chromosome scaffold as well as on whole mitotic chromosomes (34,35). 62% of the proteins that we identified as SUMO substrates in mitosis were also observed in these two studies, consistent with their association with mitotic chromosomes (Figure 2-7A). Importantly, a number of proteins identified in our study correspond to known constituents of the chromosome protein scaffold, centromeres or kinetochores, which is consistent with our detection of SUMO-2/3 at these sites on mitotic chromosomes (Figure 2-1 and Figure 2-7B).

To gain further insight into the biological functions of sumoylation and its effects and regulation in mitosis, we next analyzed the associated interaction networks and functional pathways of the identified proteins (Figure 2-8). Each protein identified is represented as a colored circle (proteins identified specifically in mitosis are red, specifically in G_0 are blue, and in both G_0 and mitosis are green), while known protein-protein interactions are shown as lines connecting the proteins (Figure 2-8). Consistent with other proteomic studies, SUMO substrates identified in our study function in a wide

range of cellular processes, including transcription, DNA repair, RNA processing and cell cycle regulation (1,32). Of note, mitochondrial proteins were enriched for in the mitotic chromosome fraction, but likely represent contaminants from co-purifying mitochondria. Of greater interest, 73% of the identified SUMO-2/3 substrates with cell cycle regulatory functions were identified in the mitotic chromosome fraction, compared to only 48% of proteins with functions in RNA processing, 55% with functions in DNA repair, and 59% of proteins with functions in transcription and chromatin remodeling.

In addition to the protein-interaction analysis, we also conducted a literature search to identify previously characterized mitotic functions for the 149 sumoylated proteins identified in association with mitotic chromosomes. Notably, more than one-third of the identified proteins (Table 2-2) regulate mitotic processes, including chromosome segregation, sister chromatin cohesion and the anaphase promoting complex. The proteins with characterized mitotic functions are represented schematically, color-coded to identify the characterized mitotic functions and connected by lines to show known protein-protein interactions (Figure 2-9). Collectively, our analysis has therefore identified >50 proteins that co-purify with mitotic chromosomes, have known mitotic functions, and are potentially regulated by SUMO-2/3 modification.

Validation of identified proteins

To provide validation that the proteins identified in our study are bona-fide SUMO-2/3 substrates, we used immunoblot analysis to investigate proteins from the same fractions used for the mass spectrometry identification. Antibodies specific for three proteins identified in the screen were used, including antibodies to topoisomerase IIα,

Trim28 (KAP-1) and KIF4A. Topoisomerase IIα was included as a positive control because it is known to be sumoylated in mitosis (12-16). Trim28, in contrast has only been shown to be sumoylated in asynchronous samples, while KIF4A has not previously been identified as a sumoylation substrate. All three proteins were detected at their predicted, unmodified, molecular weights in the starting cellular fractions. Consistent with sumoylation, all three proteins were also specifically detected in the SUMO-2/3 immunopurification at higher than expected molecular weights, but absent in the mouse IgG control (Figure 2-10). In addition to being detected in the SUMO-2/3 immunopurification from mitotic chromosomes, all three proteins were also detected in the immunopurification from G_0 nuclei.

DISCUSSION

SUMO-2/3 is detected on mitotic chromosomes throughout mitosis and is required for mitotic progression. However, the precise localization of SUMO-2/3 on chromosomes, and the identity of chromosome-associated proteins modified by SUMO-2/3, has not been fully characterized. We utilized prometaphase chromosome spreads to refine SUMO-2/3 localization to mitotic centromeres and the chromosome protein scaffold. In addition, we immunopurified endogenous SUMO-2/3 modified proteins from isolated mitotic chromosomes and G_0 nuclei and identified 244 putative sumoylated proteins by mass spectrometry. Notably, 149 proteins were detected in the mitotic chromosome fraction, and more than one-third of these putative SUMO-2/3 substrates have known functions associated with mitotic processes.

The identification of sumoylated proteins is challenging, as most proteins are modified at relatively low levels, with only 1-5% of a given protein being modified at steady state (36). This is particularly challenging in mitosis, where sumoylation is globally reduced and many regulators of mitosis are low abundance proteins (5). To enhance levels of sumoylation and substrate identification, recent proteomic analyses have identified modified proteins under conditions that cause hyper-sumoylation, including heat shock or proteasome inhibition (1,32,37,38). In addition, exogenous overexpression of tagged forms of SUMO is commonly used to enhance the abundance of sumoylated proteins, as well as to facilitate protein purification. In combination, these strategies have led to the identification of hundreds of sumoylated proteins. However, evaluating sumoylation under these experimental conditions introduces multiple caveats,

including effects of SUMO tags and overexpression on substrate specificity. Proteins modified specifically in mitosis may also be underrepresented using these approaches.

To avoid these caveats and enrich for proteins sumoylated in mitosis, we developed an immunopurification protocol that isolates endogenous chromosome-associated SUMO-2/3 modified proteins. First, cells were synchronized to obtain a population enriched in mitosis. Chromosomes were subsequently isolated to further enrich for chromosome-associated SUMO-2/3 substrates. Although a significant fraction of high molecular weight sumoylated proteins was solubilized during chromosome isolation (Figure 2-4B), whether these represent unique, soluble SUMO-2/3 substrates, or unintentionally released chromosome-associated substrates is not known. It is also notable that our fractionation strategy separated free, unconjugated SUMO-2/3 from purified chromosomes, thus reducing competition for immunopurification of low abundance sumoylated proteins (Figure 2-4B). Immunopurifications from solubilized chromosome fractions were conducted using a monoclonal antibody that recognizes SUMO-2/3 (5), and antibody-bound proteins were eluted with an epitope-specific peptide to enhance purification. As highlighted in Figure 2-6, Melchior and colleagues recently identified SUMO-1 and SUMO-2/3 modified proteins from asynchronous whole cell lysates using a related immunopurification and elution scheme (33).

In addition to immunopurifying and identifying SUMO-2/3 modified proteins associated with mitotic chromosomes, we also conducted a comparable isolation and identification of proteins from G_0 nuclei. Using these approaches, we found that more than half of the proteins identified in the mitotic chromosome fraction were also identified in G_0 nuclei. This result indicates that many proteins modified by SUMO-2/3 in

mitosis are also likely to be modified in other phases of the cell cycle. Our validation studies were consistent with this finding. Nonetheless, sumoylation may still play important functions in controlling mitosis-specific activities of the identified chromosome-associated proteins. For example, topoisomerase IIα was identified and verified to be sumoylated in both G_0 and mitotic chromosome preparations (Figure 2-6). Despite also being modified in G_0, sumoylation of topoisomerase IIα specifically at the metaphase to anaphase transition in mitosis is required for DNA decatenation and chromosome segregation (12-16). Furthermore, Ndc80 and Borealin sumoylation is specifically enriched in mitosis but low levels of sumoylation are also detected in other cell cycle phases, further suggesting that sumoylation can have cell-cycle specific functions even though substrates are sumoylated in multiple phases (19,39). Thus, all of the putative SUMO-2/3 substrates identified in our mitotic chromosome preparations warrant further investigation to understand how sumoylation may affect their mitotic functions.

In addition to the subset of proteins identified in both G_0 nuclei and associated with mitotic chromosomes, we also identified subsets of proteins specific to one of the two cell fractions. It would be intriguing to speculate that these proteins are uniquely modified in either G_0 or mitosis, but technical caveats made this interpretation difficult. Foremost, differences in synchronization and fractionation procedures complicated attempts to perform immunopurifications from truly equivalent numbers of G_0 and mitotic cells. This significantly affected the ability to quantitatively identify proteins using label-free quantification based on spectral counts. To address more definitively if identified proteins are more abundantly or uniquely modified by SUMO-2/3 in mitosis or

G_0, immunopurifications from whole cell lysates using substrate-specific antibodies, followed by SUMO-2/3 immunoblots, will need to be performed as has been demonstrated for Ndc80 and Borealin (19,39).

We identified 244 putative sumoylated proteins in G_0 and mitosis with 149 of the identified proteins being associated with mitotic chromosomes. Of interest, these findings did not reveal only a few proteins in mitosis that are massively sumoylated, but rather identified many SUMO substrates with diverse functions (Figure 2-8). Furthermore, we identified >50 proteins that have previously characterized mitotic functions ranging from chromosome alignment to regulation of anaphase initiation, highlighting the diversity of SUMO function within mitosis (Figure 2-9). For example, we identified three proteins (cell division cycle and apoptosis regulator 1 (CCAR1), ubiquitin (UBB), and anaphase promoting complex subunit 4 (ANAPC4)) involved in the structure and function of the anaphase promoting complex (APC) (40-42). Because sumoylation temporally regulates APC activation, it is intriguing to speculate that sumoylation of these three proteins is critical for APC regulation (43). Thus, this study will act as a foundation for determining the SUMO substrates responsible for the diverse mitotic functions of sumoylation.

Previous molecular and genetic studies have identified SUMO-2/3 modified proteins, including topoisomerase IIα, Pds5, and others that are enriched at centromeres and kinetochores, where sumoylation regulates sister chromatid decatenation, cohesion, and kinetochore assembly and function (11). We identified fewer than ten SUMO-2/3 modified proteins previously characterized to have centromere or kinetochore localization (Figure 2-8B). There are two possible explanations for our detection of only a few centromere and kinetochore proteins. First, centromere and kinetochore proteins

are present in very low copy numbers, so the sumoylated form of these proteins (expected to represent only a fraction of the total protein) may have been below our level of detection (44). Another possible explanation is that our synchronization method produced a predominantly prometaphase population of mitotic cells (Figure 2-3B). The exact temporal regulation of sumoylation of many kinetochore proteins is unclear, but evidence indicates that modification of topoisomerase IIα and Ndc10 is transient and limited to specific phases of mitosis (5,13,19). Proteins modified in phases of mitosis under-represented in our study may therefore have been missed.

In contrast to sumoylation at kinetochores and centromeres, little is known about how sumoylation affects the functions of proteins associated within the chromosome protein scaffold. Treating cells with the topoisomerase inhibitor, ICRF, increases levels of topoisomerase I and IIα sumoylation and the intensity of SUMO-2/3 within the protein scaffold, suggesting that the topoisomerases are a major target of scaffold sumoylation (31). Notably, we identified 10 proteins previously shown to be present in the protein chromosome scaffold by Earnshaw and colleagues including Kif4A and the block of proliferation 1 protein (Bop1), which have characterized mitotic functions (Figure 2-8B) (34). Thus, it is interesting to speculate that the requirement for Kif4A in chromosome segregation or for Bop1 in chromosome alignment and spindle morphology depends on their sumoylation in mitosis (45,46). Furthermore, we identified topoisomerase I, which localizes to mammalian mitotic chromosomes and regulates mitotic recombination and condensation of rDNA in yeast (47-49). Notably, topoisomerase 1 is also sumoylated in asynchronous cells after camptothecin treatment (50) so future analysis of topoisomerase 1 sumoylation in multiple cell cycle phases may provide insight for how sumoylation can

affect protein function in multiple cellular contexts including the mitotic chromosome scaffold.

In conclusion, we identified proteins modified in mitosis and specifically known to localize to the same mitotic structures as SUMO-2/3, namely the centromeres and kinetochores and the chromosome protein scaffold. Genetic and molecular studies have shown that sumoylation regulates sister chromatid separation, chromosome segregation, and general chromosome structure (11). However, the molecular mechanisms that demonstrate how sumoylation affects these critical mitotic functions are still largely unknown. Thus, the identification of 149 sumoylated proteins associated with mitotic chromosomes by this study will provide a foundation for investigating these molecular mechanisms in the future. Further defining the precise roles of sumoylation in mitotic progression will require targeted studies aimed at evaluating the spatial and temporal modification of individual substrates, as well as effects on localization, protein-protein interactions and/or functions.

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Figure 2-1. SUMO-2/3 localizes to the mitotic chromosome protein scaffold and centromeres. HeLa cells were treated with nocodazole for 4 hours and spun onto glass slides to produce chromosome spreads. Chromosomes were labeled using DAPI, the SUMO-2/3 antibody, and either (A) CREST, (B) phospho-histone 3 or (C) the Smc4 antibodies and analyzed by immunofluorescence microscopy. Bar = 1 μm.

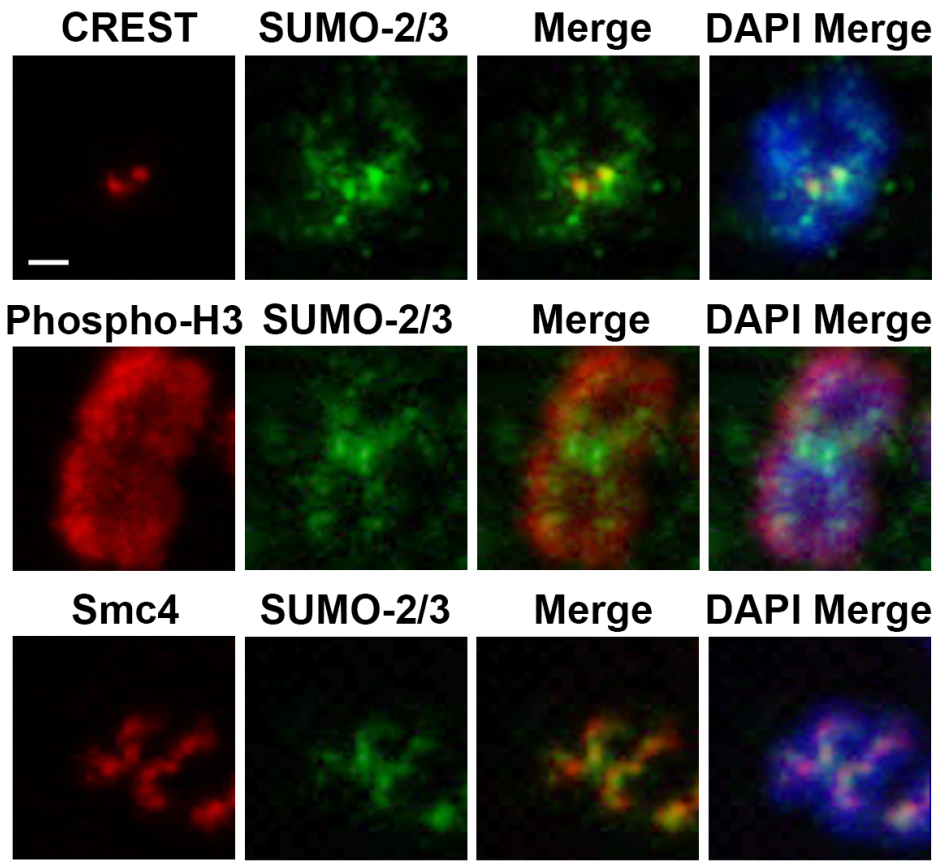

CREST
SUMO-2/3
Merge
DAPI Merge
Phospho-H3
SUMO-2/3
Merge
DAPI Merge
Smc4
SUMO-2/3
Merge
DAPI Merge

Figure 2-2. Whole chromosome spreads showing SUMO-2/3 localizes to the mitotic chromosome protein scaffold and centromeres. HeLa cells were treated with nocodazole for 4 hours and spun onto glass slides to produce chromosome spreads. Chromosomes were permeabilized with triton-X 100, fixed with paraformaldehyde and post-permeabilized with triton-x 100. Chromosomes were labeled using DAPI, the SUMO-2/3 antibody, and CREST (A), phospho-histone 3 (B) or the Smc4 (C) antibodies and analyzed by immunofluorescence microscopy. Bar = 10 μm.

CREST
SUMO-2/3
Merge
Inset
DAPI Merge
Phospho-H3
SUMO-2/3
Merge
Inset
DAPI Merge
Smc4
SUMO-2/3
Merge
Inset
DAPI Merge

Figure 2-3. Cell synchronization in G_0 and mitosis. (A) Cells were synchronized in G_0 by 24 hour serum starvation and in mitosis by an overnight nocodazole treatment and two hour release prior to harvest. DNA was stained by propidium iodide to identify condensed mitotic DNA and calculate mitotic indices. Error bars represent the standard deviation between the two independent replicates. (B) The phase distribution of the mitotic cells obtained in the mitosis preparations were counted. Each mitotic replicate is displayed individually.

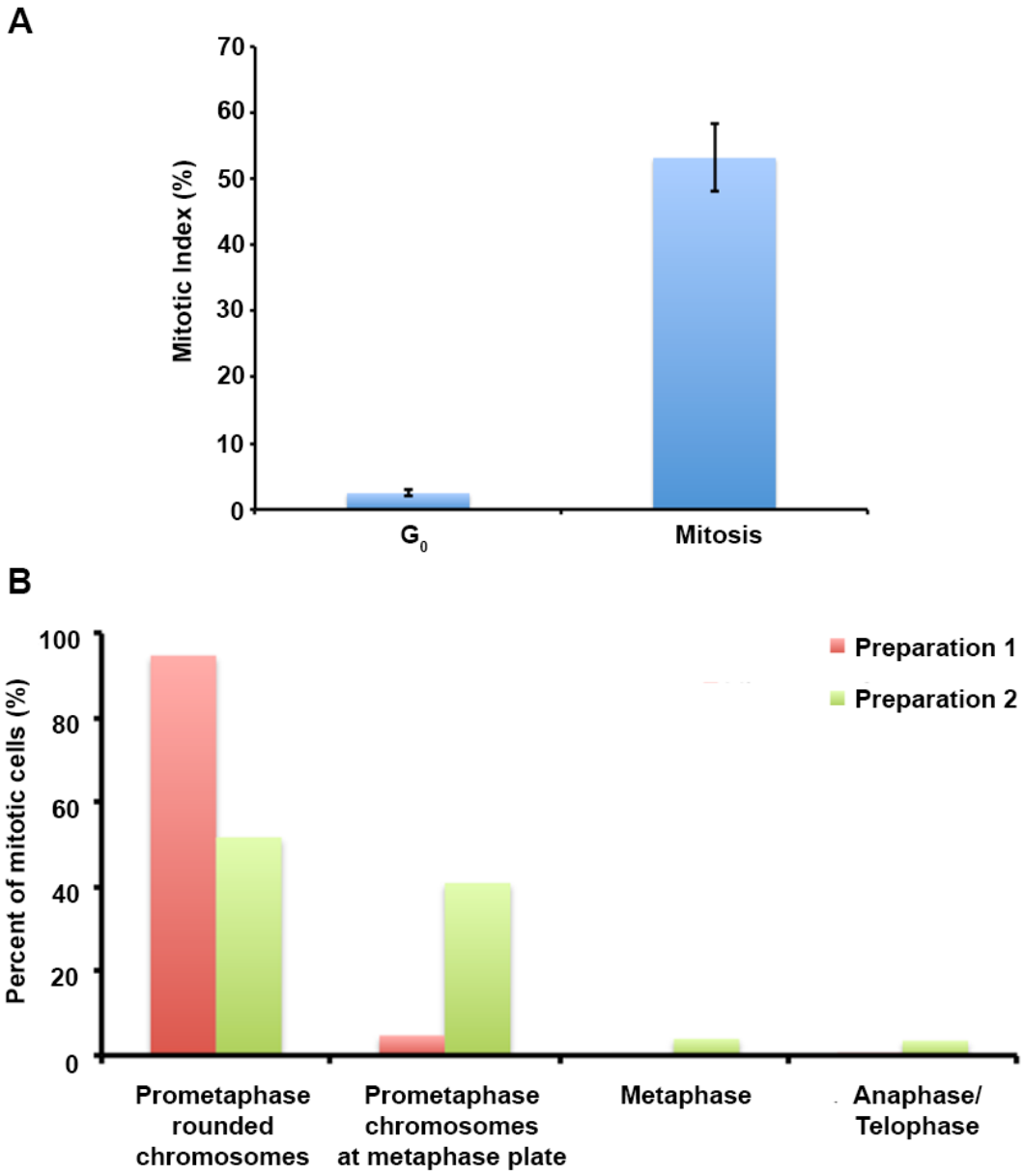

A
Mitotic Index (%)
70
60
50
40
30
20
10
0
G0
Mitosis
B
Percent of mitotic cells (%)
100
80
60
40
20
0
Preparation 1
Preparation 2
Prometaphase rounded chromosomes
Prometaphase chromosomes at metaphase plate
Metaphase
Anaphase/ Telophase

Figure 2-4. Chromosome fractionation isolates high molecular SUMO modified proteins from free SUMO-2/3. Equivalent percentages of indicated cell fractions (0.005%) were separated by SDS-PAGE (1X) and analyzed by either (A) Coomassie Blue staining or (B) immunoblotting. For coomassie blue staining, 4X of the pelleted fraction was also loaded to strengthen the signal. Immunoblotting was conducted with antibodies to heat shock protein 90 (Hsp90), histone 3 (H3) and SUMO-2/3. WCL = whole cell lysate; S = Soluble fraction; P = Pelleted fraction which represents nuclei for G_0 samples and chromosomes for mitotic samples.

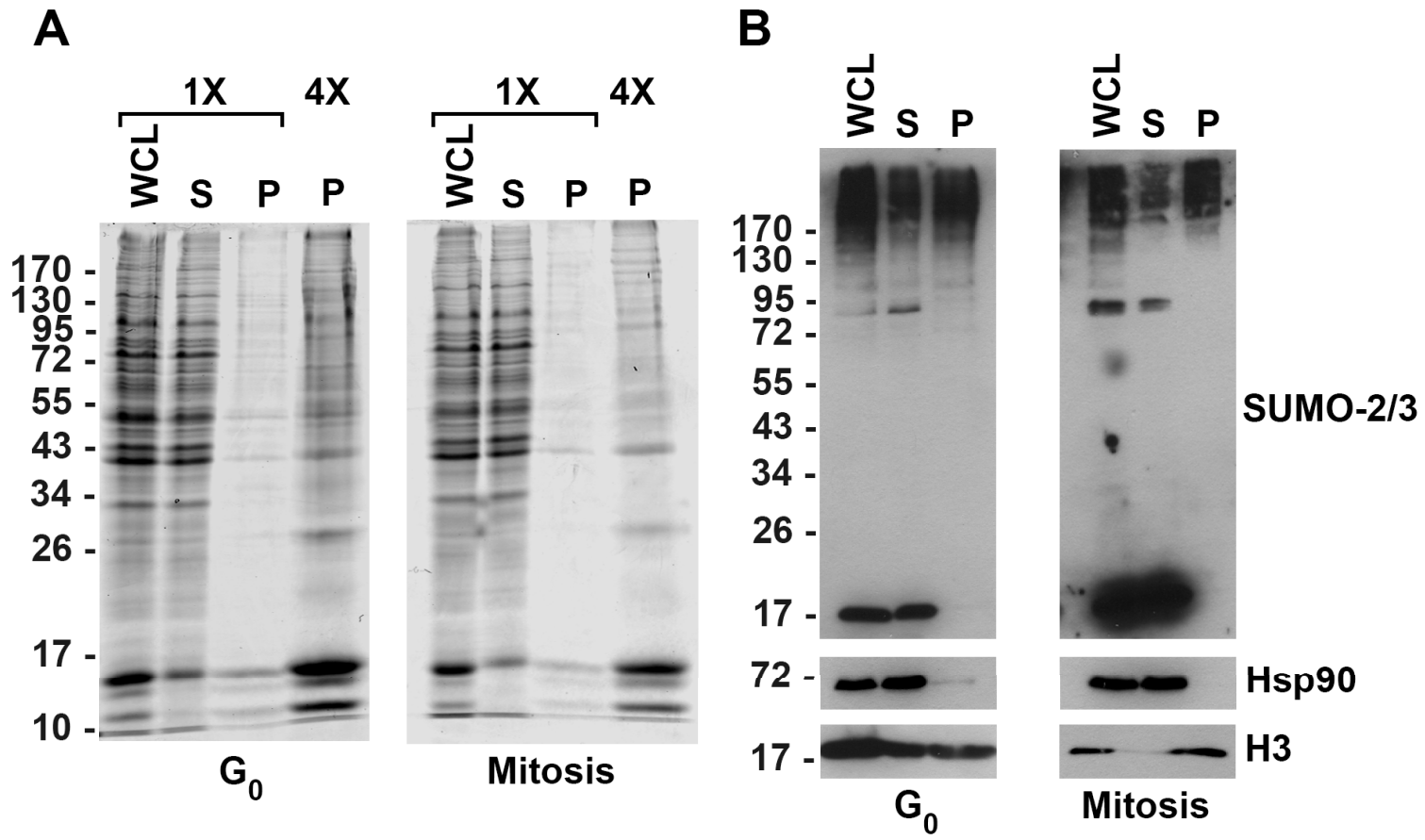

A
1X
4X
WCL
S
P
P
170
130
95
72
55
43
34
26
17
10
G0
Mitosis
B
WCL
S
P
170
130
95
72
55
43
34
26
17
72
17
SUMO-2/3
Hsp90
H3
G0
Mitosis

Figure 2-5. Immunopurification of SUMO-2/3 modified proteins from mitotic chromosomes and G_0 nuclei. (A) Schematic diagram illustrating the experimental strategy. Immunopurified proteins were eluted and equivalent percentages of each elution were separated by SDS-PAGE. Proteins were analyzed by either (B) immunoblotting with the SUMO-2/3 antibody or (C) by silver staining.

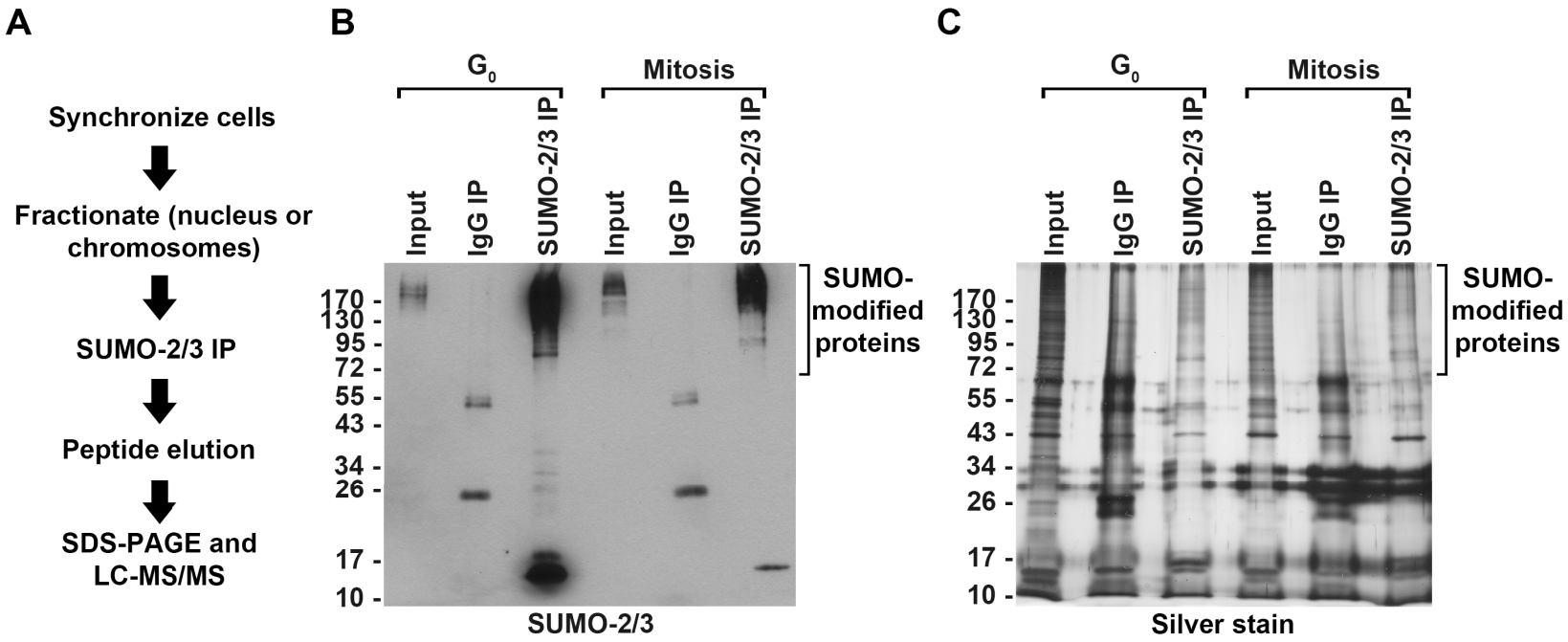

A
Synchronize cells
Fractionate (nucleus or chromosomes)
SUMO-2/3 IP
Peptide elution
SDS-PAGE and LC-MS/MS
B
G0
Mitosis
Input
IgG IP
SUMO-2/3 IP
Input
IgG IP
SUMO-2/3 IP
170
130
95
72
55
43
34
26
17
10
SUMO-modified proteins
SUMO-2/3
C
G0
Mitosis
Input
IgG IP
SUMO-2/3 IP
Input
IgG IP
SUMO-2/3 IP
170
130
95
72
55
43
34
26
17
10
SUMO-modified proteins
Silver stain

Table 2-1. Proteins identified in G_0 and mitosis by nLC-ESI-MS/MS. The gene names and protein IDs for the proteins identified in G_0 and mitosis are listed. Only proteins that had at least 2 unique peptides and a SAINT score of 0.8 MAX SAINT and 0.6 Avg SAINT are included. The total number of peptides identified for each protein in each cell cycle phase is given.

Gene Name	Protein ID	Total Peptides Identified Mitosis	Total Peptides Identified G_0
TOP2A	19913406	934	697
GTF2I	14670350	746	1353
TOP1	11225260	625	349
TOP2B	19913408	526	435
TRIM28	5032179	438	811
RANGAP1	4506411	437	401
PRKDC	13654237	347	
MGA	256017163	269	542
RSF1	38788333	250	389
SAFB2	7661936	244	599
UBB	11024714	240	351
RBM25	55741709	193	337
NFRKB	219802034	190	346
MORC3	28872812	187	238
SAFB	21264343	178	471
NOP58	7706254	177	234
MKI67	103472005	146	
ATRX	20336209	138	165
PRPF40A	151301228	128	278
CHD4	51599156	122	360
PRPF8	91208426	110	
BEND3	122937295	93	312
SART1	10863889	89	239
SUMO1	4507801	86	115
ZNF451	72255571	81	80
RANBP2	150418007	72	113
SRBD1	221136781	69	
ZMYM4	44890068	69	98
ZFP106	11968023	67	95
ARID4B	22035677	60	163
CHD3	158420731	59	105
MORC2	7662340	58	93
CHD5	24308089	58	119
ZNF800	39753953	56	35
UBA2	4885649	55	91
ANAPC4	41327749	54	208
USP39	56550051	52	143
ATP6	251831112	51	
ZNF687	24308227	48	104
YEATS2	33620755	46	84
AHCTF1	262359929	46	
NFIL3	52630429	45	53

PASD1	157785548	44	76
BRD8	34452705	44	154
KIF4A	116686122	44	15
ZBTB38	148276990	42	60
SF3B1	54112117	42	
BCLAF1	7661958	39	89
CEBPZ	42542392	37	
BAZ1B	14670392	36	
WIZ	151301215	34	83
CTCF	5729790	33	64
ZBTB4	192807284	32	37
DNMT1	195927037	32	40
ZBED1	283806699	30	
ARID4A	115334673	29	125
ZBTB20	257900533	28	30
L3MBTL2	20149698	26	70
PHIP	34996489	26	30
CUX1	148277064	25	116
UTP20	120587023	25	
ACIN1	259906018	23	82
WDR36	21281677	22	31
NUP205	57634534	21	
PWP2	48762926	20	
SURF4	19557691	20	
ZNF644	41152093	19	35
C14orf93	195233774	18	30
IARS	94721239	18	13
MBD1	156105673	17	24
CHD2	118421089	17	20
ANLN	31657094	17	
SP100	122939208	16	19
DLD	91199540	16	
PHF14	55769548	15	14
TCOF1	207113160	15	13
BOP1	21327667	15	
CTR9	7661950	15	25
HDAC1	13128860	15	
WDR3	5803221	15	22
LBR	37595750	15	
SAP130	19923597	14	41
GTF2IRD1	312836811	14	20
PNN	33356174	14	43
PDHA1	291084742	14	
HNRNPF	148470397	13	

FANCI	164607124	13	20
ZC3H14	231570121	13	36
SLC25A10	20149598	13	
EXOSC10	4505917	13	22
PBRM1	41281917	13	34
DNTTIP2	54633315	12	
DDX18	38327634	12	
KDM2B	54112380	12	18
AP2B1	4557469	12	
PES1	7657455	12	
CGNL1	31982906	12	18
PRPF4B	89276756	12	
PZP	162809334	12	10
NOLC1	148596949	11	
SLC7A5	71979932	11	
SLC27A2	227499619	11	
SSR3	6005884	11	
BRWD1	16445436	11	
WDR43	157743245	11	
DKC1	215599015	11	
BPTF	38788260	11	20
TECR	24475816	11	
ZNF295	148491088	10	
SMC3	4885399	10	
NIPBL	47578105	10	27
KPNB1	19923142	10	
NOL10	171460958	10	8
RBM28	187960109	10	
C8A	4557389	10	
CCAR1	46852388	10	
XPC	224809295	10	
TMEM33	224589127	10	7
ACAD9	21361497	10	
MCM5	23510448	10	
RLF	157671949	10	16
TMEM165	32189371	9	
TFAP2A	109389358	9	33
ZMYM2	300192959	9	11
ZBTB2	24308241	9	26
SMC1A	30581135	9	
SLC2A14	23592238	9	
SCAMP3	16445419	8	
ATP2B1	48255945	8	
CTCFL	29570785	8	

ETF1	4759034	8	7
THOC2	125656165	8	
MCM3	6631095	8	
ZMYM3	283837894	8	14
ZHX1	63079680	8	18
DHX30	20336290	8	
XPO5	22748937	8	
MSANTD4	58761537	8	28
PCNA	33239451	7	
UTP15	50980309	7	9
SRRM1	42542379	7	11
OXCT1	4557817	7	
EIF2S3	4503507	7	
INTS3	39995084	7	18
FGG	70906437	7	
NHP2	77812674	7	13
NOL11	21361468	6	7
EIF3CL	153791492	6	
PPAN-P2RY11	310923196	5	
SUMO3	48928058		214
ACTA1	4501881		175
HEATR1	73695475		80
SUPT6H	27597090		64
PRPF19	7657381		59
ZMYND8	34335262		53
RPN2	209413738		51
SYMPK	124028529		47
DDX23	41327771		37
NUP160	54859722		35
GTF3C1	101943240		34
SMCHD1	148839305		30
HDAC2	293336691		30
TRRAP	4507691		28
EEF1A2	4503475		26
USP7	150378533		22
INO80	38708321		22
DHCR7	119943112		21
DDB1	148529014		21
VDAC2	296317337		21
STAG2	112789526		21
MDC1	132626688		20
CPSF1	56676371		20
SMARCC1	188536047		19
SF3B2	55749531		19

WARS	47419914		19
PUF60	17298690		18
SIN3A	223941782		18
SMARCC2	194363725		18
SUPT5H	161169023		17
ZNF579	110681708		17
ZNF148	145386566		17
WDR75	29789283		17
VDAC3	208879465		16
HMBOX1	209180434		16
ADNP	12229217		16
KDM5B	57242796		16
POLR3D	55769552		15
RUVBL1	4506753		14
PPIG	42560244		14
ZNF263	31543980		14
CYCS	11128019		14
INTS4	50086624		14
COPB2	4758032		14
ALPI	157266292		14
PTPLAD1	117168248		14
GLYR1	40556376		13
POLR2A	4505939		13
RCOR1	7661892		13
CIZ1	196115147		13
RREB1	270132929		12
CSRP2BP	258679433		12
TP53BP1	213972634		12
KIAA1429	33946282		12
NUP188	62955803		12
SP3	289577125		12
THOC1	154448890		11
DYNC1H1	33350932		11
WDR5	16554627		10
HNRNPH3	14141157		10
CWC22	55749769		10
TRIP12	10863903		10
BAZ1A	32967603		10
RBBP4	207029415		10
DHX8	4826690		10
ZMYM6	115511046		10
ZC3H18	255652953		10
STT3A	22749415		10
HOMEZ	158631177		9

SF3A1	5032087		9
EBP	5729810		9
PCF11	33620745		9
LTBP4	110347412		9
ZFHX3	118498345		9
ACTN3	4557241		9
ZNF174	4508007		8
NNT	122939153		8
ZNF746	254553272		8
ZNF777	118918411		8
DHX38	17999539		8
MSH6	4504191		8
DDX52	38569505		8
WDR18	56243583		8
PHF3	7662018		7
DHCR24	13375618		7
CSTF3	4557495		7
BCOR	183396783		7
ESRRA	18860920		7
PAK1IP1	55769546		7
KDM1A	58761544		6
DDX56	9506931		6
PHTF2	188219565		6
ZKSCAN1	55769564		6
ZZZ3	29789072		6
BRMS1L	34147416		6

Figure 2-6. Comparison of the sumoylated proteins identified in this study to other SUMO-mass spectrometry studies. Venn diagrams comparing the results of this study to two large scale SUMO proteomic studies conducted by Ron Hay and colleagues (1,32) and a recent study by Melchior and colleagues that utilized a similar approach for immunopurifying sumoylated proteins (33).

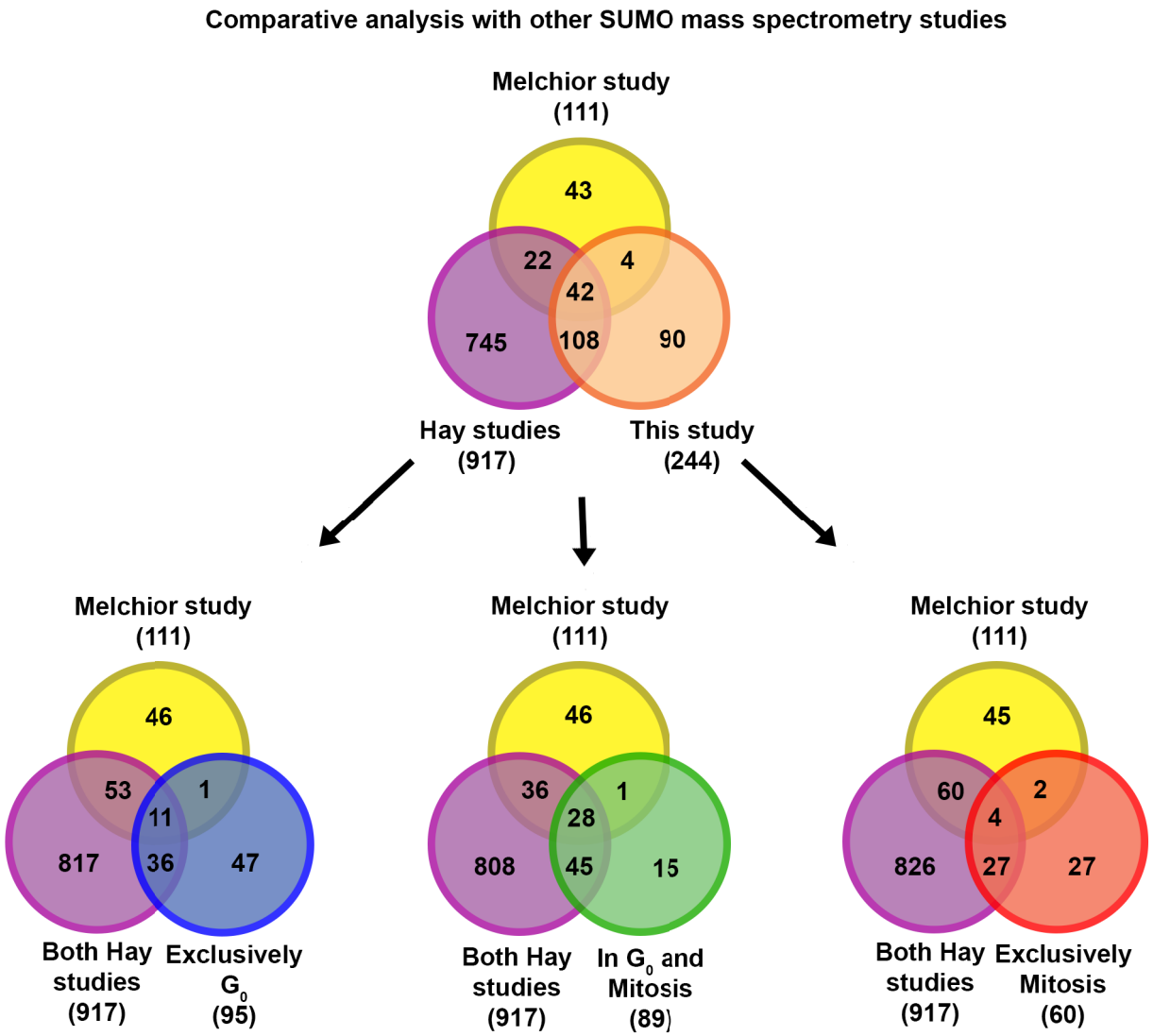
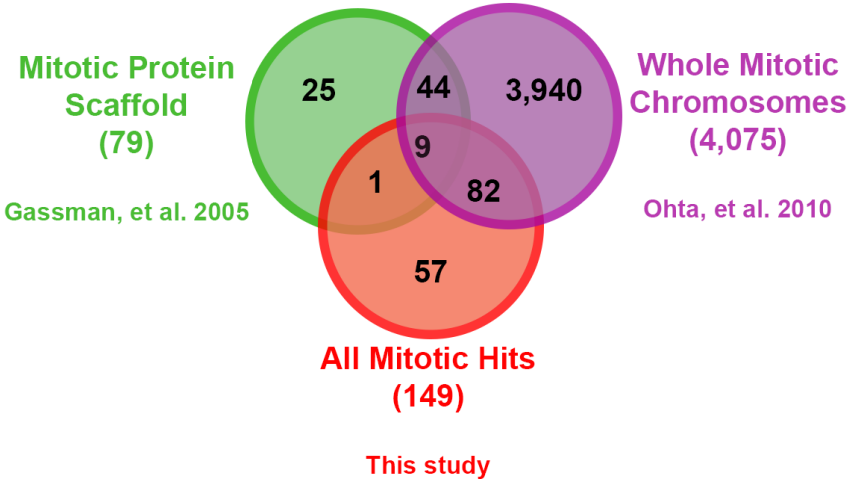

Comparative analysis with other SUMO mass spectrometry studies
Melchior study
(111)
43
22
4
42
745
108
90
Hay studies
(917)
This study
(244)
Melchior study
(111)
46
53
1
11
817
36
47
Both Hay studies
(917)
Exclusively G0
(95)
Melchior study
(111)
46
36
1
28
808
45
15
Both Hay studies
(917)
In G0 and Mitosis
(89)
Melchior study
(111)
45
60
2
4
826
27
27
Both Hay studies
(917)
Exclusively Mitosis
(60)

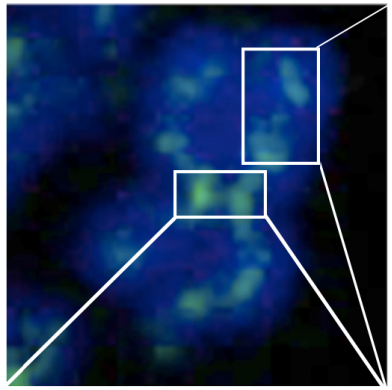
Figure 2-7. Comparison of the mitotic sumoylated proteins to other mitotic mass spectrometry studies. (A) Venn diagram comparing the results of this study to a proteomic study of the mitotic chromosome scaffold and a study of whole mitotic chromosomes (34,35). (B) A schematic showing the proteins identified in this study that are known to localize to the chromosome protein scaffold and to centromeres and kinetochores.

A

Comparative analysis with other mitotic mass spectrometry studies



B



Chromosome protein scaffold

KIF4A, PWP2, BOP1, WDR3, BAZ1B, NOL10, UTP15, NOP58, TOP2A, TOP1

Centromere/Kinetochore
RANBP2, TOP2A, TOP2B, ATRX, DNMT1, MBD1, PBRM1, RANGAP1

Figure 2-8. Interaction networks and functional pathways of the proteins identified in this study. The functions of the proteins identified in this study were determined using GO analysis and a manual literature search, which has been depicted schematically using Cytoscape software. Each circle represents a protein identified by nLC-ESI-MS/MS and the color coding describes which cell cycle phase it was identified from (Green = both G_0 and mitosis; red = specifically in mitosis; blue = specifically in G_0). The lines connecting the identified proteins represent known protein-protein interactions as documented by www.string-db.org.

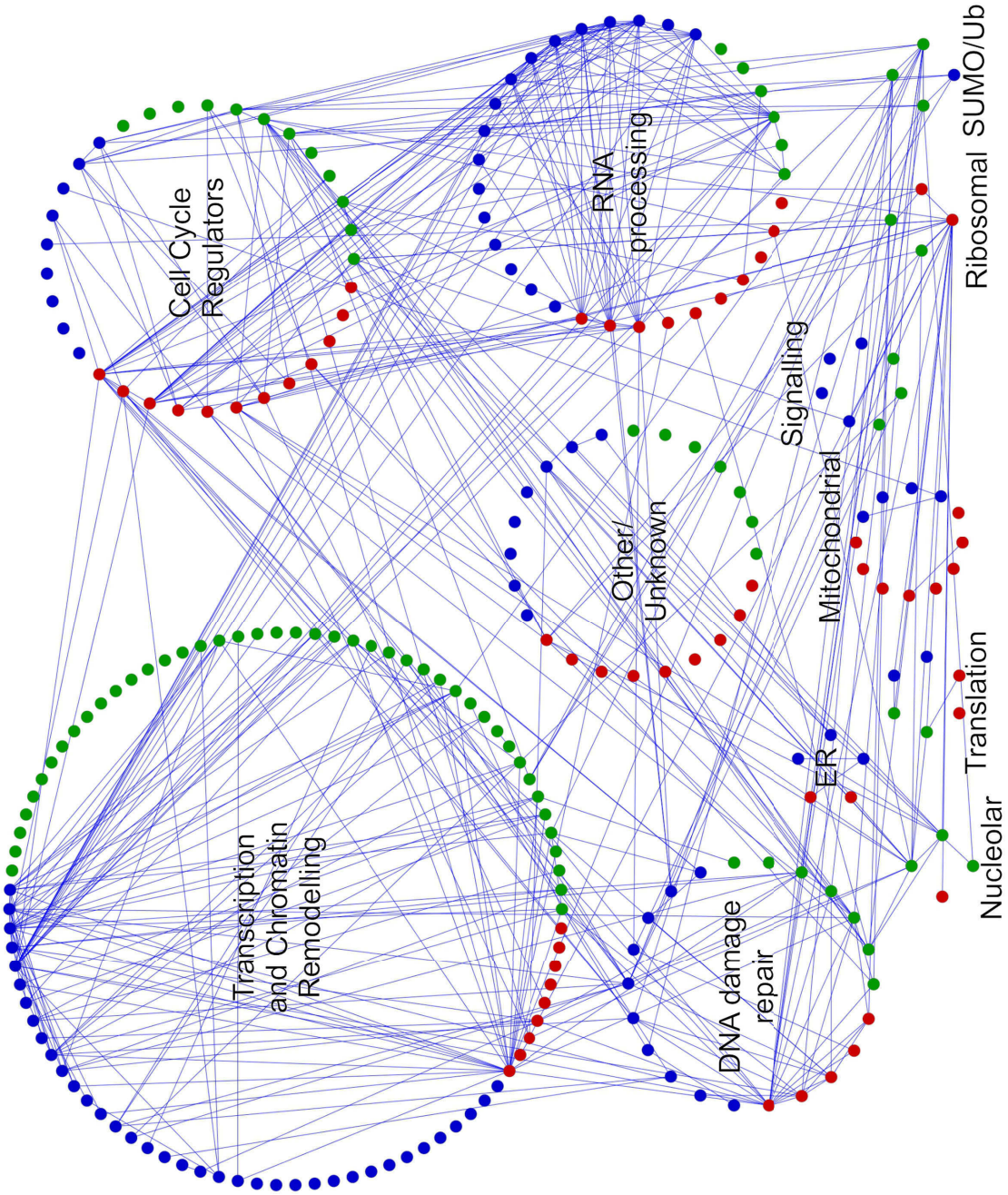

Cell Cycle
Regulators
RNA
processing
Ribosomal SUMO/Ub
Signalling
Other/
Unknown
Mitochondrial
ER
Translation
Nucleolar
DNA damage
repair
Transcription
and Chromatin
Remodelling

Table 2-2. Literature analysis describing the known mitotic functions and/or sumoylation of the proteins identified in this study as SUMO-modified in mitosis. The gene names for the 146 sumoylated proteins associated with mitotic chromosomes are listed. Known connections to sumoylation and mitosis based on literature searches are provided.

Gene Name	Mitosis	G_0	SUMO connection	Mitosis connection
TOP2A	934	697	Sumoylated (12-14,51)	DNA decatenation (52)
GTF2I	746	1353	None	None
TOP1	625	349	Sumoylated after camptothecin treatment (50)	Deletion causes increase in mitotic recombination (53)
TOP2B	526	435	Sumoylated (12-14,51)	DNA decatenation (52)
TRIM28	438	811	SUMO E3 ligase and SUMO substrate (54,55)	None
RANGAP1	437	401	SUMO substrate (22)	At kinetochores and affects spindle structure (56,57)
PRKDC	347		None	Knockdown results in chromosome alignment defects (58)
MGA	269	542	None	None
RSF1	250	389	None	Knockdown results in loss of CENP-A (59)
SAFB2	244	599	Sumoylated (60)	None
UBB	240	351	Modifies SUMO (61)	K11 chains signal from APC (40)
RBM25	193	337	None	None
NFRKB	190	346	None	None
MORC3	187	238	Sumoylation required for PML nuclear body interaction (62)	None
SAFB	178	471	Sumoylated (60)	None
NOP58	177	234	Sumoylation required for snRNA binding (63)	None
MKI67	146		None	Proliferation marker (64)
ATRX	138	165	None	At centromeres; required for cohesion and congression (65-67)
PRPF40A	128	278	None	None

CHD4	122	360	Sumoylation required for transcriptional repression (68)	Overexpression dissociated pericentrin from centrosomes (69)
PRPF8	110		None	Forms toposome with topoisomerase IIα – important for decatenation (70)
BEND3	93	312	None	None
SART1	89	239	Sumoylated at 4 sites (71)	Polymorphisms connected to cancer (72)
SUMO1	86	115	SUMO paralog	Localizes to the mitotic spindle (5)
ZNF451	81	80	Sumoylated and in PML nuclear bodies (73)	None
RANBP2	72	113	SUMO E3 ligase (74)	Knockdown causes multiple mitotic defects (56,75)
SRBD1	69		None	None
ZMYM4	69	98	None	None
ZFP106	67	95	None	None
ARID4B	60	163	Sumoylated (76)	None
CHD3	59	105	SUMO1 interacting protein by yeast 2 hybrid (77)	Overexpression dissociated pericentrin from centrosomes and knockdown disrupts centrosomes (69)
MORC2	58	93	None	None
CHD5	58	119	None	None
ZNF800	56	35	None	None
UBA2	55	91	SUMO E1 (78)	Knockdown blocks cell proliferation (79)
ANAPC4	54	208	None	Structural subunit of APC (41)
USP39	52	143	None	Required for spindle assembly checkpoint integrity and mRNA levels of Aurora B (80)
ATP6	51		None	None
ZNF687	48	104	None	None
YEATS2	46	84	None	None

AHCTF1	46		None	Required for NPC assembly at the end of mitosis (81) and required for cytokinesis (82)
NFIL3	45	53	None	None
PASD1	44	76	None	None
BRD8	44	154	None	None
KIF4A	44	15	None	Chromosome condensation and segregation (45)
ZBTB38	42	60	None	None
SF3B1	42		None	None
BCLAF1	39	89	None	None
CEBPZ	37		Alpha, beta, delta all sumoylated so most likely zeta is too (83)	Unclear; Other variants important for differentiation and proliferation (84)
BAZ1B	36		None	None
WIZ	34	83	Part of repressive complex that has 2 SUMO ligases (85)	None
CTCF	33	64	Sumoylated (86,87)	At centrosomes and then relocalizes to midbody (88)
ZBTB4	32	37	None	None
DNMT1	32	40	Sumoylated - modulates activity (89)	At centromeres (65) and knockdown arrests cells in G2 that undergo slippage and mitotic catastrophe (90)
ZBED1	30		None	On chromosomes at aligned insulators in mitosis – mitotic bookmarking (91)
ARID4A	29	125	Sumoylated (76)	None
ZBTB20	28	30	None	None

L3MBTL2	26	70	Recruitment to promoters requires sumoylation (92)	Localizes to mitotic chromosomes and overexpression causes chromosome segregation failure (93)
PHIP	26	30	None	None
CUX1	25	116	None	Hyperphosphorylated in mitosis with reduced DNA binding (94) and is required in S phase for mitotic targets (95)
UTP20	25		None	None
ACIN1	23	82	None	None
WDR36	22	31	None	None
NUP205	21		None	Knockdown negatively regulates mitotic onset (96)
PWP2	20		None	None
SURF4	20		None	None
ZNF644	19	35	None	None
C14orf93	18	30	None	None
IARS	18	13	None	None
MBD1	17	24	SUMO affects transcriptional activity (97,98)	At centromeres (65)
CHD2	17	20	None	None
ANLN	17		None	Cytokinesis (99)
SP100	16	19	Sumoylation important for PML nuclear body localization (100)	None
DLD	16		None	None
PHF14	15	14	None	None
TCOF1	15	13	None	Required for spindle orientation and localizes to centrosome and kinetochores (101)
BOP1	15		None	Knockdown results in chromosome alignment and spindle defects (46)
CTR9	15	25	None	None

HDAC1	15		Has SIM (102) and is sumoylated (103)	Specifically removed from chromosomes in mitosis to bind F-actin (104)
WDR3	15	22	None	None
LBR	15		None	Recruited to chromosomes for nuclear envelope (NE) reformation (105) and knockdown causes NE reassembly and chromosome decondensation defects (106)
SAP130	14	41	None	None
GTF2IRD1	14	20	Sumoylation affects protein-protein interactions (107)	None
PNN	14	43	None	None
PDHA1	14		None	None
HNRNPF	13		Sumoylated in proteomics and shown by IP (108)	None
FANCI	13	20	Has a SIM (109)	Localizes to fragile site loci in mitosis and are on ultra-fine DNA bridges (110,111)
ZC3H14	13	36	None	None
SLC25A10	13		None	None
EXOSC10	13	22	None	On mitotic chromosomes and knockdown causes chromosome segregation defects (112)
PBRM1	13	34	None	Localizes to kinetochores(65, 113)
DNTTIP2	12		None	None
DDX18	12		None	None
KDM2B	12	18	Ubiquitin E3 ligase (114)	None
AP2B1	12		Interacts with Ubc9 (115)	Interacts with BubR1 in spindle assembly checkpoint (116)

PES1	12		None	Localizes to periphery around metaphase chromosomes (117)
CGNL1	12	18	None	None
PRPF4B	12		None	Overexpression of N-terminus causes mitotic disruption (118)
PZP	12	10	None	None
NOLC1	11		SUMO substrate by proteomics (63)	None
SLC7A5	11		None	None
SLC27A2	11		None	None
SSR3	11		None	None
BRWD1	11		None	None
WDR43	11		None	None
DKC1	11		SUMO substrate by proteomics (63)	None
BPTF	11	20	None	None
TECR	11		None	None
ZNF295	10		None	None
SMC3	10		Complex sumoylated (119)	Cohesion (120)
NIPBL	10	27	None	Cohesion (121)
KPNB1	10		Negatively regulates RanGAP1 recruitment to kinetochores (122)	Regulates mitotic exit (123)
NOL10	10	8	None	None
RBM28	10		None	None
C8A	10		None	None
CCAR1	10		None	Binds and regulates APC (42)
XPC	10		Sumoylation stabilizes protein (124)	None
TMEM33	10	7	None	None
ACAD9	10		None	None
MCM5	10		None	Regulates centrosome duplication in S phase (125)
RLF	10	16	None	None
TMEM165	9		None	None
TFAP2A	9	33	Interacts with Ubc9 (115)	None

ZMYM2	9	11	Modified by SUMO and in PML nuclear bodies (126)	None
ZBTB2	9	26	None	None
SMC1A	9		Sumoylated (119)	Cohesion (120)
SLC2A14	9		None	None
SCAMP3	8		None	Cytokinesis (127)
ATP2B1	8		None	None
CTCFL	8		None	Suggested role coordinating S phase and mitosis (128)
ETF1	8	7	None	None
THOC2	8		None	Deletion results in mitotic recombination defects (129)
MCM3	8		SUMO interacting protein in plants (130)	Knockdown affects microtubule organization and increases multinucleated cells (131)
ZMYM3	8	14	None	None
ZHX1	8	18	Sumoylation regulates protein stability (132)	None
DHX30	8		None	None
XPO5	8		None	None
MSANTD4	8	28	None	None
PCNA	7		Sumoylation regulates DNA repair and replication (133)	None
UTP15	7	9	None	None
SRRM1	7	11	None	None
OXCT1	7		None	None
EIF2S3	7		None	None
INTS3	7	18	None	None
FGG	7		None	None
NHP2	7	13	Sumoylation validated in proteomics (63)	None
NOL11	6	7	None	None
EIF3CL	6		None	None
PPAN-P2RY11	5		None	None

Figure 2-9. Previously characterized mitotic functions of the sumoylated proteins associated with mitotic chromosomes. Schematic representation of the >50 proteins that have previously characterized mitotic functions. Each circle represents a mitotic SUMO-substrate identified in this study. The color-coding denotes the mitotic function associated with that protein and the lines connecting the identified proteins represent known protein-protein interactions as documented by www.string-db.org with a cutoff confidence level of 0.7.

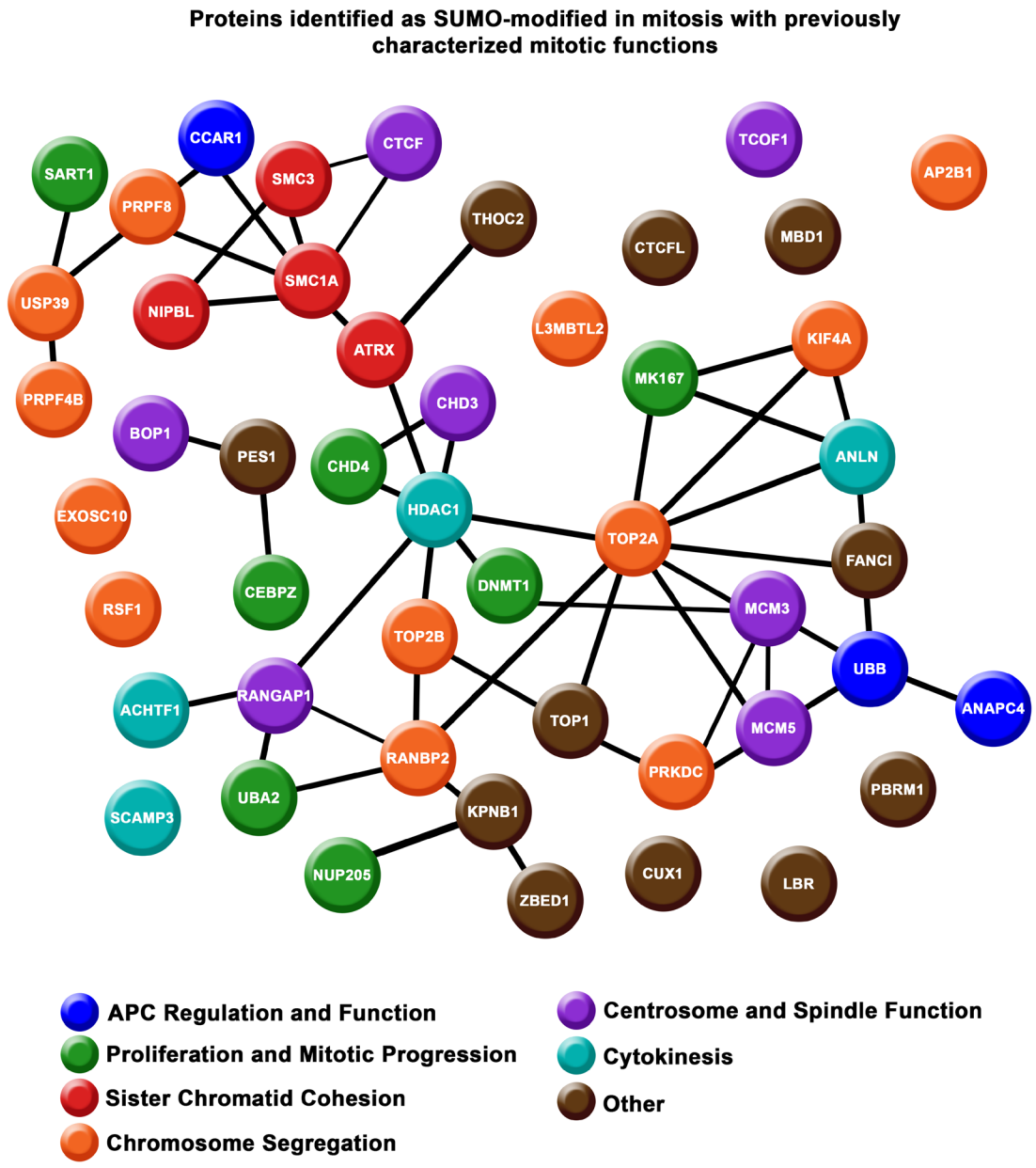
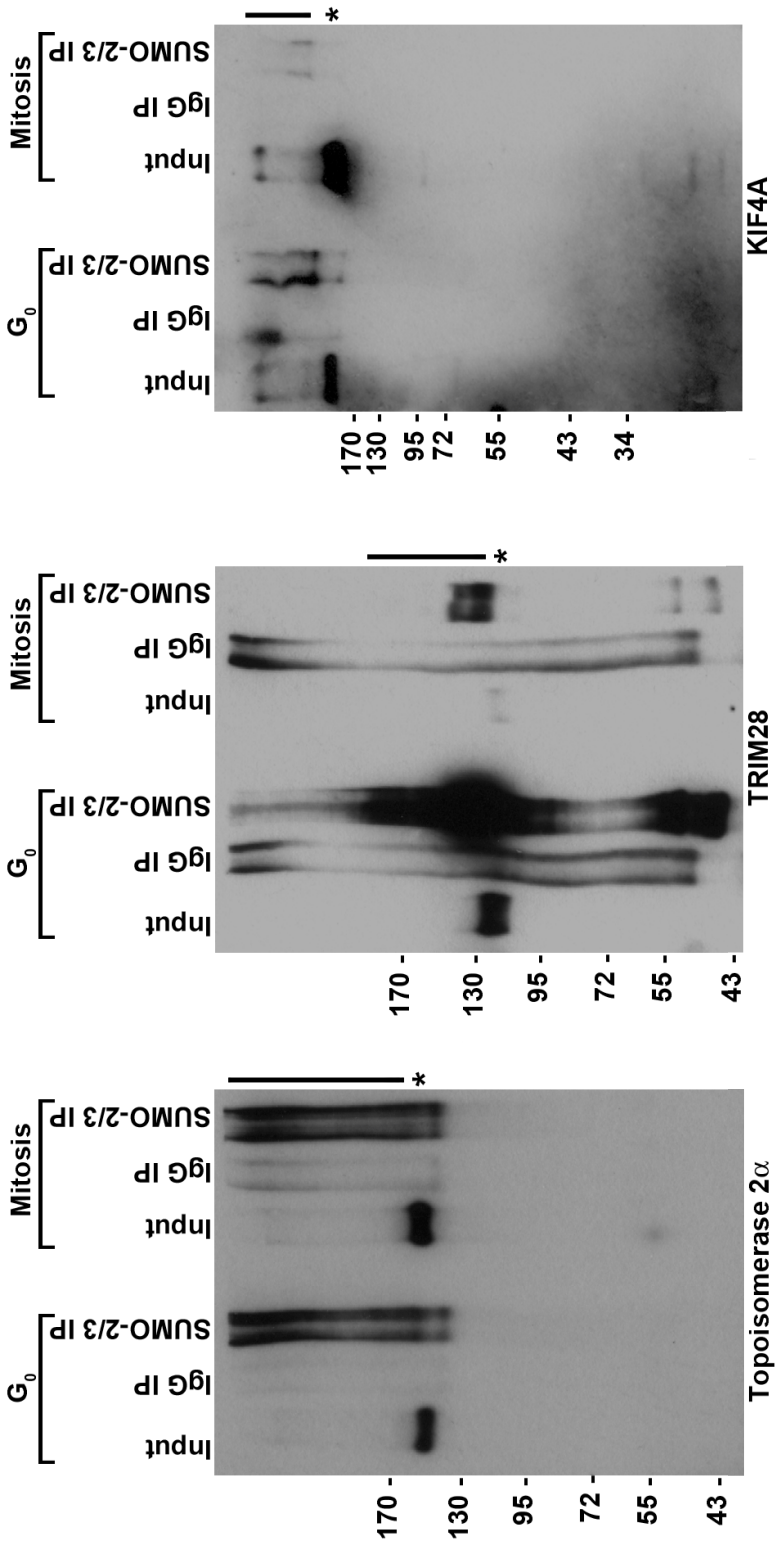

Proteins identified as SUMO-modified in mitosis with previously characterized mitotic functions
CCAR1
CTCF
SART1
SMC3
PRPF8
THOC2
TCOF1
AP2B1
CTCFL
MBD1
SMC1A
USP39
NIPBL
L3MBTL2
KIF4A
ATRX
MK167
PRPF4B
CHD3
BOP1
PES1
CHD4
ANLN
EXOSC10
HDAC1
TOP2A
FANCI
CEBPZ
DNMT1
RSF1
MCM3
TOP2B
UBB
ACHTF1
RANGAP1
TOP1
MCM5
ANAPC4
RANBP2
PRKDC
PBRM1
UBA2
SCAMP3
KPNB1
NUP205
CUX1
LBR
ZBED1
APC Regulation and Function
Proliferation and Mitotic Progression
Sister Chromatid Cohesion
Chromosome Segregation
Centrosome and Spindle Function
Cytokinesis
Other

Figure 2-10. Validation of identified sumoylated proteins. SUMO-2/3 modified proteins were immunopurified from G_0 nuclei and chromosome fractions and analyzed by immunoblotting with antibodies to topoisomerase IIα, Trim28, and KIF4A as indicated. Equivalent fractions of proteins purified by mouse IgG (IgG IP) and SUMO-2/3 immunopurifications (SUMO-2/3 IP) are included. The astericks marks the molecular weight of the unmodified protein and the line highlights the sumoylated forms of the protein.


Topoisomerase 2α
TRIM28
KIF4A
Mitosis
G0
Input
IgG IP
SUMO-2/3 IP
170
130
95
72
55
43
34
*

CHAPTER 3

SENP1 AND SENP2 AFFECT SPATIAL AND TEMPORAL CONTROL OF SUMOYLATION IN MITOSIS

ABSTRACT

Sumoylation of centromere, kinetochore, and other mitotic chromosome-associated proteins is essential for chromosome segregation. The mechanisms regulating spatial and temporal sumoylation of proteins in mitosis, however, are not well understood. Here, we show that the SUMO-specific isopeptidases, SENP1 and SENP2, are targeted to kinetochores in mitosis. SENP2 targeting was found to occur through a mechanism dependent on the Nup107-160 subcomplex of the nuclear pore complex (NPC), and to be modulated through interactions with karyopherin α. Overexpression of SENP2, but not other SUMO-specific isopeptidases, caused a defect in chromosome congression that was dependent on its precise kinetochore targeting. By altering SENP1 kinetochore associations, however, this effect on chromosome congression could be phenocopied. In contrast, RNAi-mediated knockdown of SENP1 delayed sister chromatid separation at metaphase, whereas SENP2 knockdown produced no detectable phenotypes. Collectively, our findings indicate that chromosome segregation is dependent on precise spatial and temporal control of sumoylation in mitosis, and that SENP1 and SENP2 are important mediators of this control.

INTRODUCTION

Regulation of essential mitotic processes is achieved in large measure through the action of posttranslational protein modifications, including phosphorylation, ubiquitylation and sumoylation. Phosphorylation has been particularly well studied, as some of the best characterized regulators of kinetochore and microtubule interactions possess protein kinase activity, including the Aurora kinases and BUBR1 (1,2). Ubiquitylation also plays a number of well established roles in controlling mitotic progression, in particular by facilitating proteasome-mediated degradation of proteins including securin and the mitotic cyclins (3). Sumoylation represents a more recently discovered regulator of mitosis, and multiple studies have already revealed essential roles in controlling chromosome condensation and cohesion, kinetochore assembly and function, and spindle dynamics (4-15). The molecular targets and mechanisms of action of sumoylation during mitosis, however, still remain to be fully explored.

Small ubiquitin related modifiers (SUMOs) are ~100 amino acid proteins that, like ubiquitin, are covalently conjugated to lysine residues in substrate proteins (16,17). Invertebrates express a single SUMO protein while vertebrates express three predominant SUMO paralogs: SUMO-1, SUMO-2 and SUMO-3. SUMO-2 and SUMO-3 are highly related, sharing 96% sequence homology, and are therefore referred to collectively as SUMO-2/3. SUMO-1 shares only 45% similarity to SUMO-2/3. Biochemical and proteomic analyses have identified distinct subsets of proteins that are modified uniquely by SUMO-1 or SUMO-2/3, indicating that SUMO paralogs may regulate unique biological processes and have distinct signaling properties (17,18). Of particular interest, SUMO-1 and SUMO-2/3 are uniquely regulated and conjugated to distinct proteins

during mitosis (13). The molecular mechanisms regulating the spatial and temporal control of paralog-selective modifications and functions, however, are also not well understood.

Sumoylation occurs through a three-step enzymatic cascade, which requires the concerted action of an ATP-dependent E1 activating enzyme (Aos1/Uba2 heterodimer), an E2 conjugating enzyme (Ubc9) and one of a number of SUMO-specific E3 ligases (16,19). Although regulation of substrate modification can occur at the level of conjugation, regulation at the level of desumoylation by SUMO-specific isopeptidases also plays an important role. Yeast express two major SUMO-specific isopeptidases, Ulp1 (Ubiquitin-like protease) and Ulp2, while vertebrates express six enzymes referred to as SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7 (20). These enzymes possess conserved C-terminal catalytic domains and divergent N-terminal domains that determine subcellular localization and substrate selectivity (21-28). In addition to being localized to distinct subcellular domains, the six vertebrate SENPs also exhibit differences in SUMO paralog specificity. This specificity is determined by differences in both N-terminal and C-terminal domains (29,30).

Given the unique activities and localizations of the vertebrates SENPs, they represent potentially important spatial and temporal regulators of sumoylation in cells. Consistent with this, SENP2 and SENP6 have both been implicated in having important, but functionally distinct, roles in regulating sumoylation in mitosis. SENP6 affects kinetochore assembly by limiting sumoylation of the kinetochore-associated protein, CENP-I, and its degradation through the SUMO-targeted ubiquitin E3 ligase, RNF4 (11). SENP2 is believed to regulate sumoylation of kinetochore-associated proteins necessary

for the association of CENP-E with kinetochores. We previously demonstrated that the recruitment of CENP-E to kinetochores was dependent on its ability to interact non-covalently with SUMO2/3, and that SENP2 overexpression resulted in a loss of CENP-E from kinetochores (13). How the substrate recognition and function of SENP2 and SENP6 are controlled spatially and temporally in mitosis is not understood, but of great interest.

Here, we present evidence that SENP1 and SENP2 are positioned to uniquely exert spatial and temporal control on sumoylation of proteins in mitosis. We show that SENP2 is distinct from SENP1 and SENP6 in its ability to cause a mitotic, prometaphase arrest when overexpressed in cultured mammalian cells. The ability of SENP2 to cause a cell cycle arrest was due to a unique association with kinetochores during prophase that was dependent on interactions with the Nup107-160 subcomplex of the NPC and karyopherin α. We also found that SENP1 associates with the mitotic spindle and kinetochores in mitosis, but had no effect on mitotic progression when overexpressed. In contrast to overexpression phenotypes, RNAi-mediated knockdown of SENP1 prevented timely separation of sister chromatids at the metaphase to anaphase transition. Together, our findings reveal critical and non-redundant roles for SENP1 and SENP2 in mitosis, and demonstrate the importance that sub-cellular localization plays in defining SUMO mammalian isopeptidase function.

MATERIALS AND METHODS

Antibodies

SENP2 and GFP rabbit polyclonal antibodies were produced as described previously (31). SENP1 antibody, a gift from Dr. Mary Dasso (National Institute of Health, Bethesda, MD), was generated by injecting rabbits with GST-SENP1 (273-449) as previously described (32). Antibodies were affinity purified using appropriate antigens and standard protocols.

Remaining antibodies were obtained from the following sources: anti-Nup107 antibody was kindly provided by Dr. Joseph Glavy (Charles V. Schaefer, Jr. School of Engineering & Science, New York, NY); CREST human auto-antibodies were a generous gift from Dr. Ted Salmon (University of North Carolina, NC); anti-karyopherin α3 was provided by Dr. Stephen Adams (Northwestern University, Chicago, IL); anti-CENP-E (Active Motif, Carlsbad, CA); anti-GFP (Clontech, Mountain View, CA); anti-INCENP (Active Motif, Carlsbad, CA); anti-HEC1 (BD Biosciences, San Jose, CA); anti-Tubulin (Sigma-Aldrich, St. Louis, MO); anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA); anti-FLAG M2 (Sigma-Aldrich, St. Louis, MO); mAb414 (Abcam, Cambridge, MA); anti-phospho-histone H3 (Ser10) (EMD Millipore, Billerica, MA).

Plasmid constructs

SENP2 cDNA and GFP-tagged expression constructs were obtained as previously described (23,31). GFP-SENP1 and YFP-SENP6 vectors were a gift from Dr. Mary Dasso (National Institute of Health, Bethesda, MD). To generate SENP1 and SENP2

chimeric protein expression constructs, site-directed mutagenesis was used to create restriction sites at the junction between the N-terminal domain and the catalytic domain of both SENP1 and SENP2. The catalytic domain of one isopeptidase was PCR-amplified and ligated into this restriction site to replace the original catalytic domain. Flag-Nuf2 was subcloned into pC_4-R_HE vector for mammalian expression (Ariad Pharmaceuticals, Cambridge, MA). SENP1 was subcloned into the pC_4EN-F1 vector (Ariad Pharmaceuticals, Cambridge, MA). SENP1 was subcloned into a pmCherry-C2 vector and the siRNA resistant clones and catalytic mutants were generated using site-directed mutagenesis.

Cells, cell culture, transfection, RNA interference

HeLa cells stably expressing YFP-Histone H2B were a gift from Dr. Andrew Holland (Johns Hopkins University School of Medicine, Baltimore, MD). HeLa or 293T cells were maintained at 37°C in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 10 mM HEPES (pH 8.0) and 1% penicillin-streptomycin. Cells were transfected with the indicated plasmids at a confluency of 40-50% using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For RNA interference, cells were grown to 40-50% confluency and then transfected using RNAiMax (Invitrogen, Carlsbad, CA). siRNA oligos were used at a final concentration of 20 nM. siRNA oligos included: scramble control (5'-CUUCCUCUCUUUCUCUCCCUUGUGA-3'); SENP2 oligo(a) (5'-AUAUCUGGAUUCUAUG GGAUU-3'); SENP2 oligo(b) (5'-GAAAGAGAGAAGUACCGAAtt-3'); SENP1 oligo(a) (5'-

UCCUUUACACCUGUCUCGAUGUCUU-3') and SENP1 oligo(b) (5'-GCAAAUGGCCAAUGG AGAAAUUCUA-3'). Cells were harvested for immunoblotting, immunofluorescence microscopy, or timelapse microscopy 48 hours after transfection. For co-transfection of siRNA oligos and siRNA resistant forms of SENP1, cells were grown to 40-50% confluency, transfected using Lipofectamine 2000 using a final concentration of siRNA oligo of 20 nM.

Heterodimerization

For heterodimerization experiments, cells were transfected with indicated plasmids and cultured in the presence or absence of 250 nM AP21967 for 48 hr (Ariad Pharmaceuticals, Cambridge, MA) prior to analysis by immunofluorescence microscopy. For immunopurification, cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1% Triton-X 100, 1% sodium deoxycholate, 2 mM EDTA, 1 μg/ml leupeptin and pepstatin A, 20 μg/ml aprotinin, 2 mM PMSF. Lysates were placed on ice for 5 min and then sonicated for 30 sec and centrifuged at 16,000 x g for 15 min. Lysates were incubated with M2 Flag agarose beads (Sigma, St. Louis, MO) for 4 hr at 4°C and then beads were washed 6 times in PBS and bound proteins were eluted directly in SDS-sample buffer.

GFP-SENP immunopurifications

For GFP-SENP immunopurifications, rabbit anti-GFP antibodies were immobilized on Protein-A Plus agarose beads (Thermo Scientific, Rockford, IL) for one hour and crosslinked with DSS for 30 minutes. Beads were washed through a series of

four buffers including 50 mM Tris pH = 7.5, 100 mM glycine pH = 3.0, PBS, and lysis buffer (25 mM Tris pH = 7.4, 150 mM NaCl, 1% NP-40, 1 mM DTT, 0.05% sodium deoxycholate). 293T cells were transfected with the indicated plasmids for approximately 36 hrs, treated with or without 0.1 μg/ml nocodazole overnight and then harvested 48 hr after transfection. Cells were lysed in lysis buffer supplemented with 1 mM PMSF, 5 ug/ml pepstatin A, 5 ug/ml leupeptin and 10 mM N- ethylmaleimide, sonicated and centrifuged 16,000 x g for 20 minutes at 4ºC. Protein lysates were quantified using a BCA protocol (Thermo Scientific, Rockford, IL) to normalize protein inputs. Antibody-bound beads were incubated with cell lysates for 5 hours at 4ºC, washed 6 times with lysis buffer, and proteins were eluted directly in SDS-sample buffer.

Immunoblotting

Immunoblot analysis was performed using enzyme-linked chemiluminescence ECL-Prime reagent (GE Healthcare, Silver Spring, MD).

Immunofluorescence microscopy and live cell imaging

HeLa cells were cultured on glass coverslips. Unless otherwise stated, cells were fixed in 2% formaldehyde for 30 min and permeabilized in 0.2% Triton-X 100 for 7 min at RT. For colocalization with kinetochore proteins, cells were fixed in 3.5% paraformaldehyde in PBS for 7 min and permeabilized in 0.5% Triton-X 100 in PBS for 20 min at RT. Localization of GFP- SENP1 was examined by pre-extracting in 20 μg/ml digitonin in buffer containing (200 mM HEPES (pH 6.5), 110 mM potassium acetate, 20 mM magnesium acetate, 1 μg/ml leupeptin and pepstatin A, 20 μg/ml aprotinin, and 1

mM PMSF) for 15 min at RT and then fixing in 2% formaldehyde in PBS for 30 min. Immunostaining was carried out as previously described using secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Life Technologies, Grand Island, NY), unless otherwise noted (13). Images were acquired using the Zeiss Observer Z1 fluorescence microscope with a Zeiss Plan-Apochromat 63X objective (numerical aperature = 1.40) and an Apotome VH optical sectioning grid. Images were obtained using a Zeiss Axiocam MRm camera and processed using AxioVision Software Release 4.8.2 (Zeiss, Gena, Germany).

For live cell imaging, cells were cultured in Lab-Tek Chambered #1.0 Borosilicate Coverglass slides (Nunc, Rochester, NY), transfected, and then imaged 48 hrs post-transfection. For imaging, cells were maintained in culture medium at 37ºC with 5% CO_2 on a Zeiss Observer Z1 fluorescence microscope fitted with an incubation chamber. Images were acquired using a Zeiss EC Plan-Neofluar 40X objective (numerical aperature = 1.3) every 5 minutes for 15 hours with a Zeiss Axiocam MRm camera.

RESULTS

SENP2 overexpression uniquely induces prometaphase arrest

We previously demonstrated that overexpression of SENP2 in cultured mammalian cells results in a global reduction in sumoylation and a prometaphase arrest phenotype caused by defects in the targeting of CENP-E to kinetochores (13). Because mammalian cells express multiple different SUMO-specific isopeptidases with distinct subcellular localizations and enzymatic activities, we sought to investigate whether overexpression studies could be used to identify functions for other SENPs in mitosis.

We transiently transfected constructs encoding GFP-SENP1, GFP-SENP2, or YFP-SENP6 together with Myc-tagged SUMO-2 into HeLa cells and first examined the effects of overexpressing these isopeptidases on sumoylation. SENP1 was chosen for comparison due to its close similarity to SENP2, and SENP6 due to previous evidence of mitotic functions (11). In comparison to control cells, overexpression of all three isopeptidases caused comparable global decreases in the levels of high molecular weight SUMO-2 conjugates (Figure 3-1A). To determine whether the suppression of sumoylation caused by SENP1, SENP2, or SENP6 overexpression resulted in similar prometaphase arrest phenotypes, we analyzed the cell cycle distribution of cells 48 hrs following transfection. As expected, we observed a reproducible 2-fold increase in the mitotic index of SENP2 overexpressing cells, with a majority of mitotic cells arresting specifically in prometaphase with lagging chromosomes concentrated at the spindle poles (Figure 3-1B, D and E). In contrast, cells overexpressing SENP1 or SENP6 showed no noticeable defects in chromosome segregation and the fraction of cells in mitosis were comparable to those of control cells (Figure 3-1B, D and E). Notably, the ability of

SENP2 overexpression to cause a prometaphase arrest was dependent on suppression of SUMO-2 conjugate levels, as overexpression of a catalytically inactive mutant ($SENP2_{C548A+W457A}$) did not affect levels of SUMO-2/3 modified proteins (Figure 3-1A) and had no effect on cell cycle progression (Figure 3-1B).

SENP2-induced prometaphase arrest is determined by its N-terminal domain

The ability of SENP2 overexpression to uniquely affect mitotic chromosome segregation could be explained by distinct enzymatic activity, as determined by its C-terminal catalytic domain, or distinct sub-cellular localization or substrate specificity, as established by elements in its N-terminal domain (20,30,31). To distinguish between these two possibilities, we generated expression constructs for chimeric SENP1 and SENP2 proteins. The N-terminal domain of SENP1 was fused to the catalytic domain of SENP2 to generate $SENP1_N\text{-}2_{CAT}$ and the N-terminal domain of SENP2 was fused to the catalytic domain of SENP1 to generate $SENP2_N\text{-}1_{CAT}$ (Figure 3-1C). Constructs coding for these chimeric proteins were transfected into cells together with Myc-tagged SUMO-2 and their effects on sumoylation were monitored by immunoblot analysis. Overexpression of $SENP1_N\text{-}2_{CAT}$ and $SENP2_N\text{-}1_{CAT}$ both caused global decreases in high molecular weight SUMO-2 conjugates similar to those obtained with overexpression of wild-type SENP1 and SENP2 (Figure 3-1A). To evaluate effects on cell cycle progression, we also analyzed transfected cells by fluorescence microscopy and quantified mitotic indices. Overexpression of $SENP2_N\text{-}1_{CAT}$ caused an arrest of cells in prometaphase with chromosomes concentrated at the spindle poles, similar to the arrest observed with wild-type SENP2 (Figures 1B, 1D and 1E). Consistent with this effect,

overexpression of SENP2 and the $SENP2_N$-1_{CAT} chimera both blocked recruitment of CENP-E to kinetochores, as previously reported for SENP2 (13) (Figure 3-1E). In contrast, and similar to wild-type SENP1, overexpression of the $SENP1_N$-2_{CAT} chimera had no noticeable effect on cell cycle progression, chromosome segregation, or CENP-E kinetochore targeting (Figure 3-1B, D and E). Thus, the N-terminal domain of SENP2 contains unique determinants critical to its ability to affect CENP-E targeting and prometaphase arrest.

The N-terminal domains of SENP1 and SENP2 direct overlapping and unique subcellular localizations in interphase and mitosis

Because the N-terminal domains of SUMO-specific isopeptidases are important determinants of localization, we next examined the subcellular distributions of GFP-SENP1, GFP-SENP2, as well as the chimeric proteins in interphase and mitosis. SENP1 and SENP2 were detected predominantly at the nuclear envelope and NPCs during interphase, as previously reported or suggested (23-25,32) (Figure 3-2). As predicted based on previous studies of SENP2 NPC targeting (31), the localization $SENP2_N$-1_{CAT} chimera was indistinguishable from wild type SENP2 (Figure 3-2B). Similarly, the localization of the $SENP1_N$-2_{CAT} chimera was also indistinguishable from wild type SENP1 (Figure 3-2B). Because the SENP2 catalytic domain alone contains no NPC-targeting signals (31), this result is consistent with signals for NPC localization also residing within the N-terminal domain of SENP1.

To evaluate localizations in mitosis, cells transfected with GFP-SENP1 and GFP-SENP2 were labeled with human CREST antibodies to mark the centromeres of mitotic

chromosomes. SENP1 was detected at centrosomes, along spindle microtubules, as well as at foci partially co-localizing with CREST, indicative of kinetochore localization (Figure 3-3A). SENP2 was detected in nondescript aggregates, but also at foci partially co-localizing with CREST. Unlike SENP1, SENP2 was not detected at centrosomes or on spindle microtubules (Figure 3-3A). Co-localization studies with INCENP and Hec1, markers for the inner-centromere and outer-kinetochore respectively, further confirmed and narrowed the association of SENP1 and SENP2 to elements of the outer-kinetochore (Figures 3B and 3C). The $SENP1_N$-2_{CAT} and $SENP2_N$-1_{CAT} chimeras showed localization patterns in mitotic cells indistinguishable from wild-type SENP1 and SENP2, respectively (Figure 3-3A). Collectively, these localization studies demonstrate that the N-terminal domains of SENP1 and SENP2 specify overlapping and distinct associations with NPCs in interphase and with elements of the mitotic spindle and kinetochore in mitosis.

SENP1 and SENP2 both associate with the Nup107-160 subcomplex of the NPC but interact differentially karyopherin α

The association of SENP2 with NPCs is mediated in part through interactions with the Nup107-160 subcomplex and karyopherins (31). Both interactions also have the potential to affect targeting to kinetochores in mitosis (33). However, SENP2 interactions in mitotic cells have not previously been characterized, and interactions mediating SENP1 association with NPCs also have not been fully investigated. We therefore performed immunopurifications of GFP-tagged SENP1 and SENP2 from lysates prepared from synchronized cells arrested in mitosis, as well as from

asynchronous cells. Interactions with karyopherin α3 and Nup107 were investigated by immunoblot analysis. Both karyopherin α3 and Nup107 co-purified with SENP2 from asynchronous and mitotic cell lysates (Figure 3-4). Nup107 also co-purified with SENP1 in immunopurifications from both mitotic and interphase lysates, consistent with stable association with the Nup107-160 subcomplex (Figure 3-4). However, unlike SENP2, stable interactions between SENP1 and karyopherin α3 were not detected. These findings are consistent with the overlapping and distinct distributions of SENP1 and SENP2 in interphase and mitosis.

Two N-terminal elements of SENP2 specify kinetochore association and are required for prometaphase arrest

The Nup107-160 subcomplex of the NPC localizes to kinetochores in mitosis (33). To determine whether interactions with the Nup107-160 subcomplex facilitate SENP2 targeting to kinetochores, and whether this targeting is required for prometaphase arrest, HeLa cells were transfected with a construct encoding GFP-tagged SENP2 Δ144-349 (Figure 3-5A). This SENP2 deletion mutant is defective in interactions with the Nup107-160 subcomplex (31). Although overexpression of SENP2 Δ144-349 caused a decrease in high molecular weight SUMO-2 conjugates similar to overexpression of wild-type SENP2 (Figure 3-5B), no detectable effect on cell cycle progression was observed based on quantitative analysis of mitotic indexes (Figure 3-5C). Consistent with a functional relationship between kinetochore localization and prometaphase arrest, SENP2 Δ144-349 exhibited a diffuse cytoplasmic distribution in mitotic cells that was devoid of kinetochore or spindle association (Figure 3-5D).

A second NPC-targeting element in SENP2 consists of a nuclear localization signal (NLS) within the N-terminal 63 amino acids that mediates high affinity interactions with karyopherin α (31). To evaluate the role of karyopherin α binding in affecting SENP2 localization in mitosis, we next transfected HeLa cells with SENP2 Δ1-63, a deletion mutant lacking the N-terminal 63 amino acids (Figure 3-5A). Similar to wild-type SENP2, overexpression of SENP2 Δ1-63 resulted in global decreases in high molecular weight SUMO-2 conjugates as revealed by immunoblot analysis of whole cell lysates (Figure 3-5B). Based on analysis of mitotic indices, however, overexpression of SENP2 Δ1-63 failed to produce a noticeable effect on cell cycle progression (Figure 3-5C). Surprisingly, analysis by fluorescence microscopy revealed that SENP2 Δ1-63 was detectable at kinetochores, but also at centrosomes and along spindle microtubules, in a fashion mirroring SENP1 localization (Figure 3-5D). These findings demonstrate that the association of SENP2 with kinetochores in mitosis requires interactions with the Nup107-160 subcomplex, and that karyopherin α binding restricts SENP2 localization. Karyopherin α, however, was not detected at kinetochores in SENP2 overexpressing cells (Figure 3-6). The results also demonstrate that the effect of SENP2 overexpression on mitotic progression correlates with restricted kinetochore localization.

Tethering SENP1 to kinetochores induces a prometaphase arrest

We hypothesized that a more stable association with kinetochores, or association with distinct kinetochore-associated proteins, could underlie the unique ability of SENP2 to affect mitotic progression. To test this hypothesis, we investigated the effects of artificially tethering SENP1 to kinetochores using the rapamycin-based

heterodimerization system (34,35). Specifically, we fused the ligand-binding domain of the FK506 binding protein (FKBP) to HA-tagged SENP1 and the rapamycin-binding domain of the FKBP-rapamycin-associated protein (FRB) to Flag-tagged Nuf2, an outer-kinetochore protein (Figure 3-7A). Cells were either transiently transfected with a construct encoding FRB-Flag-Nuf2 alone, or together with a construct encoding HA-SENP1-FKBP. Transfected cells were then incubated in the presence or absence of the heterodimerizer, AP21967. To demonstrate effective AP21967-mediated heterodimerization, FRB-Nuf2 was immunopurified from transfected cell lysates using a Flag-specific antibody and immunoblot analysis was performed using an HA-specific antibody. As expected, co-purification of SENP1-FKBP with FRB-Nuf2 was dependent on both co-expression and the presence of the AP21967 heterodimerizer. Minimal interaction between SENP1-FKBP and FRB-Nuf2 was detected in the absence of AP21967 (Figure 3-7B).

To examine how heterodimerization with FRB-Nuf2 affects SENP1-FKBP localization, we examined the distributions of both fusion proteins in co-transfected cells cultured in the absence or presence of AP21967. Under the specific fixation and permeabilization conditions used, SENP1-FKBP was detected as a diffuse signal throughout mitotic cells in the absence of heterodimerizer, with no appreciable detection at kinetochores (Figure 3-7C). Under similar assay conditions, FRB-Nuf2 was detected in distinct foci that co-localized with the outer-kinetochore protein CENP-E (Figure 3-7C). When cells were cultured in the presence of heterodimerizer, SENP1-FKBP was detected in prominent foci throughout mitotic cells that co-localized with FRB-Nuf2, consistent with enhanced kinetochore localization (Figure 3-7C).

To characterize the effects of artificially tethering SENP1 to the outer-kinetochore, we next analyzed the cell cycle distribution of co-transfected cells cultured in the absence or presence of heterodimerizer (Figure 3-7D). Expression of either FRB-Nuf2 or SENP1-FKBP alone did not significantly affect cell cycle progression, irrespective of the presence or absence of AP21967. Co-expression of both fusion proteins caused a 2-3 fold increase in the mitotic index when cells were cultured in the absence of the heterodimerizer, possibly reflecting the low level of basal interaction detected by immunopurification (Figure 3-7B and D). Most notably, however, a 5-6 fold increase in the mitotic index was detected in cells co-expressing FRB-Nuf2 and SENP1-FKBP and cultured in the presence of AP21967. Importantly, this effect on cell cycle progression was dependent on SUMO deconjugation, as heterodimerization of a catalytically inactive SENP1 mutant (SENP1 C603A) with FRB-Nuf2 had significantly reduced effects on the mitotic index relative to effects observed in the absence of heterodimerizer (Figure 3-7B and 7D). Similar to the phenotype observed with SENP2 overexpression, tethering SENP1 to Nuf2 at the outer-kinetochore resulted in the accumulation of prometaphase-arrested cells, many of which exhibited lagging chromosomes present at the spindle poles (Figure 3-7E). These results support a model whereby the inhibitory effects of SENP2 overexpression on mitotic progression are mediated through precisely localized deconjugation of SUMO-modified proteins at kinetochores.

RNAi depletion reveals a critical function for SENP1 in mitosis

The findings outlined above involved characterization of exogenously expressed SENP1 and SENP2. To evaluate endogenous proteins, we performed immunofluorescence microscopy with SENP1 and SENP2 specific antibodies. As previously reported, SENP2 specific antibodies weakly stained the nucleoplasm and cytoplasm and were concentrated at the nuclear envelope in interphase cells (Figure 3-8A) (31). To evaluate co-localization with kinetochores in mitosis, cells were co-labeled with antibodies specific for Hec1. Although SENP2 antibodies labeled foci associated with condensed mitotic chromosomes, we were unable to definitively localize endogenous SENP2 to kinetochores due to limitations of the antibody and SENP2 expression levels (Figure 3-8B). Large GFP-SENP2 foci observed in interphase and mitotic cells were not observed when analyzing endogenous SENP2. Consistent with GFP-SENP1 localization, antibodies specific for SENP1 labeled predominantly the nuclear envelope in interphase cells, with a slight nucleoplasmic signal also being detected (Figure 3-8A). In mitotic cells, SENP1 was detected as diffuse puncta throughout cells, with additional concentrations observed at mitotic spindles (Figure 3-8B and C). As with SENP2, definitive localization at kinetochores was not possible.

To further explore the functions of endogenous SENP1 and SENP2 in mitosis, we finally turned to RNAi to knock down protein expression in HeLa cells. In HeLa cells, SENP2 is present as multiple isoforms ranging from 45 to 60 kDa that are thought to arise from alternative pre-mRNA splicing (31). Each of these SENP2 isoforms was reduced by >90% using two independent siRNAs (Figure 3-9A). SENP1 specific antibodies detected a predominant band of ~70 kDa, that was also reduced by >90%

using two independent siRNAs (Figure 3-9A). The effects of SENP1 or SENP2 depletion on cell cycle progression were analyzed by fluorescence microscopy and quantitative assessment of mitotic indices. This analysis revealed no detectable effect on cell cycle progression in SENP2-depleted cells (Figure 3-9B). In SENP1 depleted cells, a reproducible 2 to 3 fold increase in mitotic index was observed relative to control cells using both independent siRNAs (Figure 3-9B).

To characterize effects on mitotic progression in greater detail, we repeated SENP1 and SENP2 depletions in HeLa cells expressing YFP-tagged histone H2B. We utilized live-cell imaging to measure the time between nuclear envelope breakdown (NEBD) and metaphase alignment, as well as metaphase alignment to initiation of anaphase (Figure 3-9C and D). Consistent with the mitotic index assessment, SENP2-depleted cells did not exhibit an increase in the time spent in mitosis compared to the control cells (Figure 3-9C and D). In contrast, although SENP1-depleted cells progressed from NEBD to metaphase normally, they exhibited a clear delay in anaphase onset following metaphase alignment (Figure 3-9C and D). SENP1-depleted cells ultimately completed cell division despite prolonged times in metaphase, demonstrating that the SENP1-depletion conditions resulted in a mitotic delay but not an arrest. To validate that this effect was SENP1-specific, we co-transfected cells with siRNAs and constructs coding for siRNA-resistant forms of wild type or catalytically inactive (C603A or C603S) mCherry-SENP1 and repeated the live-cell image analysis. Each of the mCherry-SENP1 proteins was expressed at comparable levels, while endogenous SENP1 expression was clearly reduced (Figure 3-9E). As predicted, wild type SENP1 expression restored normal metaphase to anaphase kinetics, while the catalytically inactive forms of SENP1

failed to do so (Figure 3-9F). These results demonstrate that SENP1, and its isopeptidase activity, is critical for timely metaphase to anaphase transition. Collectively, the findings reveal that SENP1 and SENP2 depletions produce distinct results from SENP1 and SENP2 overexpression and reveal a critical, non-redundant role for SENP1 in chromosome segregation.

DISCUSSION

Sumoylation is essential for chromosome segregation in organisms ranging from yeast to humans (36,37). This requirement is related in part to spatial and temporal regulation of kinetochore assembly, including the association of CENP-E and the CENP-H/I/K complex with kinetochores during prophase in mammalian cells (11,13). In this study we have provided evidence that the SUMO-specific isopeptidases, SENP1 and SENP2, are positioned to affect spatial and temporal control of sumoylation through unique associations with kinetochores, spindle microtubules and centrosomes. Consistent with roles in affecting spatial and temporal control of sumoylation in mitosis, manipulating the expression levels of SENP1 or SENP2 induced defects in chromosome congression in prometaphase or sister chromatid separation at metaphase. Importantly, observed overexpression phenotypes correlated with the precise sub-cellular localizations of SENP1 and SENP2, demonstrating the crucial connection between isopeptidase targeting and biological function.

Our studies to elucidate the localizations of SENP1 and SENP2 in mitosis relied largely on analysis of exogenously expressed GFP-tagged proteins. A number of lines of evidence indicated that the mitotic localizations that we observed with GFP-SENP1 and GFP-SENP2 accurately reflect the localizations of the endogenous proteins. First, both proteins were found to associate with the Nup107-160 subcomplex, whose localizations to spindle microtubules and kinetochores in mitosis are well established. In the case of SENP2, we confirmed that its association with kinetochores was dependent on interactions with the Nup107-160 subcomplex through analysis of SENP2 Δ144-349 mutant. Although we were unable to obtain definitive evidence for endogenous SENP2

at kinetochores using immunofluorescence microscopy, this likely reflected the combination of low SENP2 expression levels and the relatively small fraction of the Nup107-160 subcomplex that is targeted to kinetochores (33). In contrast to SENP2, our immunofluorescence analysis revealed that a fraction of SENP1 is enriched at the mitotic spindle, consistent with the observed localization of GFP-SENP1. Determining whether SENP1 localization is dependent on the Nup107-160 complex, however, will require additional studies.

Previous studies have established that signals in the N-terminal domains of mammalian SENPs specify their subcellular localizations (23-26,31). Consistent with this, we found using chimeric fusion proteins that the N-terminal domains of SENP1 and SENP2 determine their unique associations with NPCs, kinetochores and spindle microtubules. The N-terminal domain of SENP2 contains multiple elements that mediate association with NPCs during interphase, including an element that binds the Nup107-160 subcomplex of the NPC and an element that binds karyopherins (31). Our findings indicated that both of these elements also contribute to the association of SENP2 with kinetochores in mitosis. SENP1 was also found to associate with the Nup107-160 subcomplex, but was distinct from SENP2 in that stable associations with karyopherin α were not detected.

The Nup107-160 subcomplex redistributes from NPCs to spindle poles, microtubules and the outer kinetochore plate in mitosis, consistent with a role in mediating SENP1 and SENP2 localizations to these structures (33,38-40). The Nup107-160 subcomplex plays important roles in controlling mitotic events, including chromosome segregation, by affecting the distribution of Aurora B and other

chromosome passenger complex (CPC) proteins (41). Notably, several members of the CPC are sumoylated, including Aurora B and Borealin (27,42,43). Our findings that SENP1 and SENP2 are both associated with the Nup107-160 subcomplex suggest the intriguing possibility that its effects on CPC distribution may be related in part to control of CPC sumoylation.

Although SENP1 localized to the mitotic spindle and kinetochores, the distribution of SENP2 was distinct in its more restricted localization to kinetochores. The restricted localization of SENP2 was dependent on interactions with the Nup107-160 subcomplex, but also depended on interactions mediated by N-terminal residues that include a functional NLS. This NLS mediates high affinity interactions with karyopherin α, and in particular, RanGTP-insensitive interactions with karyopherin α3 (31). Deletion of the N-terminal NLS caused SENP2 to localize to spindle microtubules as well as kinetochores, a distribution comparable to the localization of the Nup107-160 subcomplex and SENP1. This change in localization could be explained by higher levels of soluble SENP2 Δ1-63 relative to full-length SENP2. However, detection of spindle staining for both proteins was unaffected by expression levels. Based on current knowledge, it is therefore hypothesized that both SENP1 and SENP2 are targeted to spindle microtubules and kinetochores through interactions with the Nup107-160 subcomplex, and that interactions with karyopherin α function to further stabilize or restrict SENP2 localization at kinetochores. Further studies are required to test this hypothesis.

Importantly, the mitotic arrest phenotype observed upon SENP2 overexpression was dependent on the more restricted, karyopherin α-dependent, localization of SENP2.

We interpret this finding as an indication that the restricted localization of SENP2 at kinetochores affects its substrate selectivity and thereby acts to distinguish SENP2 from SENP1. By facilitating kinetochore association, karyopherin α could affect SENP2 substrate selectivity by enhancing its local concentration at kinetochores. Alternatively, karyopherin α could function to target SENP2 to a distinct subdomain of the kinetochore, or more directly facilitate association with specific SUMO-modified proteins. Our finding that SENP1 could be detected at kinetochores when overexpressed, but exerted no mitotic defects unless artificially tethered to Nuf2, is consistent with the interpretation that the precise localizations or concentrations of SENP1 and SENP2 dictate their substrate selectivity and function. Identification of the SUMO-modified proteins recognized by SENP1 and SENP2 in mitosis will help to better clarify how localization, concentration, or other parameters affect substrate specificity.

How karyopherin α affects the association of SENP2 with kinetochores is unclear, but karyopherins in general have a number of established roles in affecting protein localization during mitosis (44). Of particular interest, the relative distribution of hKid between spindle microtubules and chromosome arms is determined by interactions with karyopherin α and β. In a manner reminicient of our findings for SENP2, interactions with karyopherin α and β function to restrict hKid localization to spindle microtubules while promoting interactions with chromosome arms (45). Also of potential relevance, studies in budding yeast have demonstrated a mitotic role for the karyopherin Kap121p in the transport of Ulp1 from NPCs to the septin ring (46). Further studies are required to determine the precise molecular details of how karyopherins affect protein

targeting in mitosis, including the targeting and dynamic association of SENP2 with kinetochores.

In addition to providing evidence of mitotic roles for SENP1 and SENP2 in mitosis using protein overexpression studies, we also made important observations in cells depleted of SENP1 or SENP2. We observed a reproducible 2-3 fold increase in the mitotic index upon depletion of endogenous SENP1. Our timelapse microscopy analysis demonstrated that this increase is due to a delay in the separation of sister chromatids following normal alignment at the metaphase plate. Furthermore, we demonstrated that this effect is due to the loss of SENP1 isopeptidase activity, as only a wild type siRNA-resistant SENP1 rescued this phenotype. Although further studies are required to define the precise defect, a delay in the metaphase to anaphase transition may be related to improper desumoylation of a protein involved in the spindle assembly checkpoint or dissolution of sister chromatid cohesion. Surprisingly, gene knockout studies in chicken DT40 cells revealed a requirement for SENP1 in the maintenance of sister chromatid cohesion in the presence of microtubule destabilizing agents (47). Although our SENP1 RNAi studies revealed no defects in cohesion maintenance, differences in findings could be explained by the unique experimental conditions, including the presence and absence of drug treatments, transient knockdown versus knockout, and human HeLa cells versus chicken DT40 cells. In contrast to SENP1, timelapse microscopy did not identify any obvious mitotic defects in SENP2 depleted cells, which could be explained in a number of ways. First, SENP2 may be redundant with other SENPs, including SENP3, which has been shown to affect desumoylation of Borealin (27). Alternatively, hyper-sumoylation

of mitotic proteins resulting from SENP2 depletion may have limited functional consequences, in contrast to hypo-sumoylation resulting from SENP2 overexpression.

Our evaluations of the overexpression and knockdown phenotypes of SENP1 and SENP2 have revealed critical roles for both sumoylation and desumoylation at multiple points during mitosis. Our findings have also highlighted the importance that subcellular localization plays in defining the activities of SENP1 and SENP2 in mitosis and their unique and non-redundant functions. Ultimately, the identification of SENP1 and SENP2 mitotic substrates will be essential to more fully understand the role that these enzymes play in regulating chromosome segregation and, more generally, how sumoylation controls this vital cellular process.

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Figure 3-1. SENP2 overexpression uniquely affects mitotic progression through mechanisms dependent on its N-terminal domain. (A) HeLa cells were co-transfected with constructs coding for Myc-tagged SUMO-2 and the indicated GFP-tagged SUMO isopeptidases, or empty vector (Mock) as control. Cell lysates were analyzed by immunoblot analysis with antibodies specific for Myc, GFP or tubulin. **(B)** HeLa cells were transfected with constructs coding for the indicated GFP-tagged SUMO isopeptidases or empty vector (Mock). The fraction of transfected cells in mitosis was determined by fluorescence microscopy 48 hrs following transfection. **(C)** Schematic diagram of SENP1, SENP2 and SENP1/2 chimeras. CAT=catalytic domain. **(D)** HeLa cells were transfected with constructs coding for the indicated SUMO isopeptidases or empty vector (Mock). The fraction of transfected cells present at each of the indicated stages of mitosis was determined by fluorescence microscopy 48 hrs following transfection. **(E)** HeLa cells were transfected with constructs coding for SENP1, SENP2, or the indicated chimeras. Cells were stained with CENP-E specific antibodies and analyzed by immunofluorescence microscopy. DNA was stained with DAPI. Arrowheads indicate unaligned chromosome pairs. Bar = 10 μm. Where indicated, error bars represent standard deviations from three independent experiments.

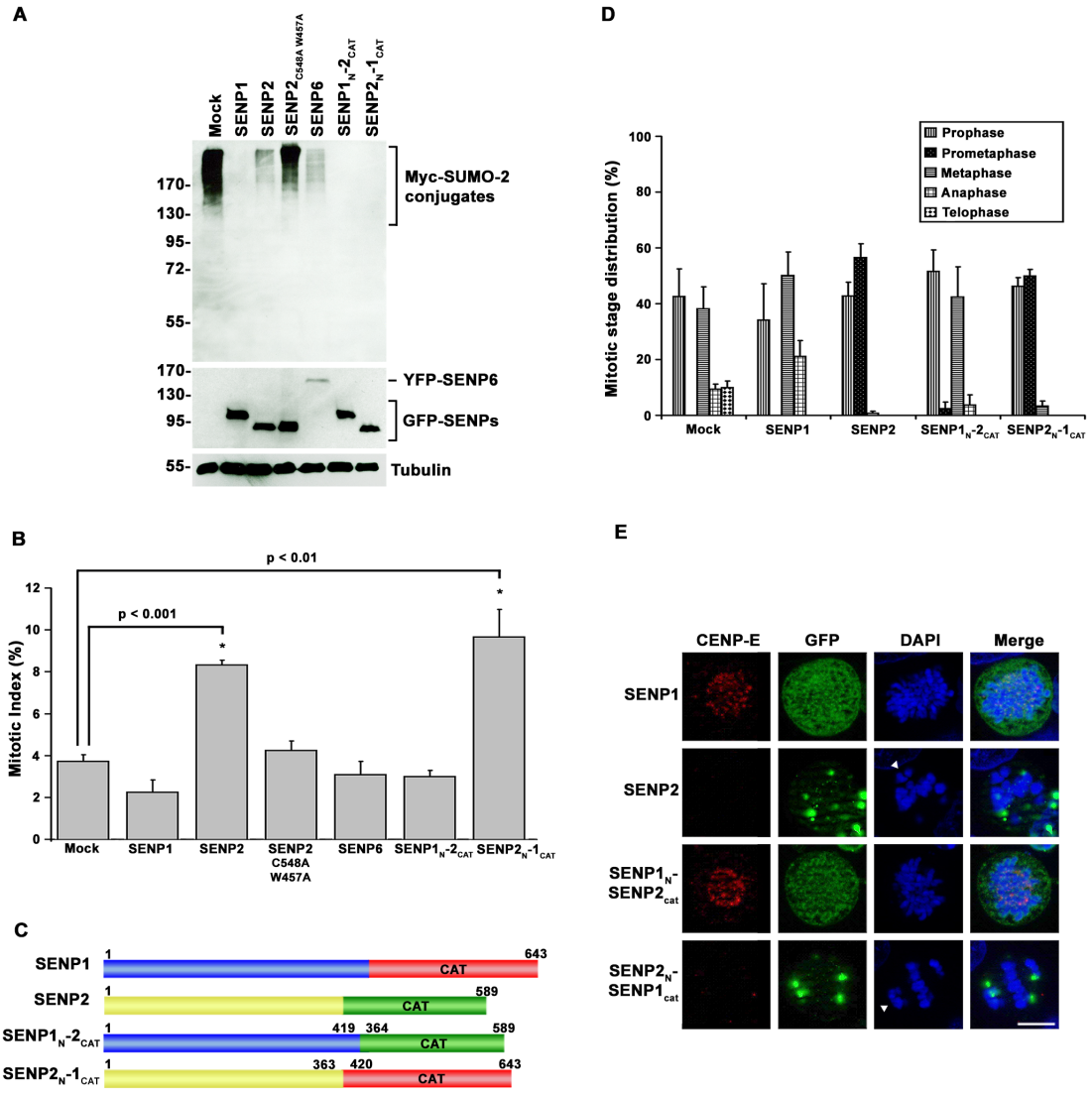

A
Mock
SENP1
SENP2
SENP2C548A W457A
SENP6
SENP1N-2CAT
SENP2N-1CAT
170-
130-
95-
72-
55-
Myc-SUMO-2 conjugates
170-
130-
95-
YFP-SENP6
GFP-SENPs
55-
Tubulin
B
p < 0.01
p < 0.001
Mitotic Index (%)
12
10
8
6
4
2
0
Mock
SENP1
SENP2
SENP2 C548A W457A
SENP6
SENP1N-2CAT
SENP2N-1CAT
C
SENP1
1
643
CAT
SENP2
1
589
CAT
SENP1N-2CAT
1
419
364
589
CAT
SENP2N-1CAT
1
363
420
643
CAT
D
Mitotic stage distribution (%)
100
80
60
40
20
0
Prophase
Prometaphase
Metaphase
Anaphase
Telophase
Mock
SENP1
SENP2
SENP1N-2CAT
SENP2N-1CAT
E
CENP-E
GFP
DAPI
Merge
SENP1
SENP2
SENP1N-SENP2cat
SENP2N-SENP1cat

Figure 3-2. **SENP1 and SENP2 association with NPCs is determined by N-terminal targeting signals.** HeLa cells were transfected with constructs coding for GFP-tagged SENP1, SENP2, or the indicated chimeras, fixed with formaldehyde and permeabilized with TNX-100. Cells were stained with mAb 414 to detect nuclear pore complexes and imaged by immunofluorescence microscopy. DNA was stained with DAPI. Bar = 10 μm.

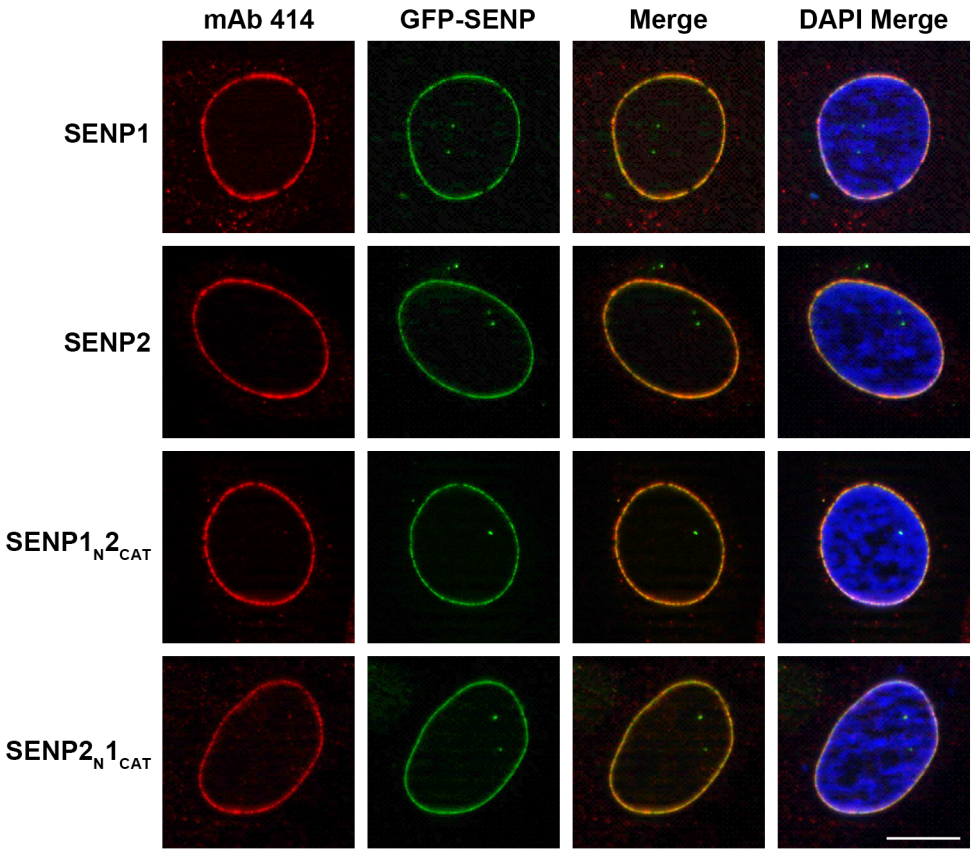

mAb 414
GFP-SENP
Merge
DAPI Merge
SENP1
SENP2
SENP1N2CAT
SENP2N1CAT

Figure 3-3. SENP1 and SENP2 are targeted to overlapping and distinct mitotic structures through their N-terminal domains. Constructs coding for GFP-tagged SENP1, SENP2 or SENP1/2 chimeras were transfected into HeLa cells. Cells expressing SENP1 or $SENP1_N2_{cat}$ were permeabilized, fixed and stained, while cells expressing SENP2 or $SENP2_N1_{cat}$ cells were fixed, permeabilized and stained. Cells were co-labeled with: **(A)** CREST anti-centromere antibodies. Asterisks highlight centrosome staining and arrowheads indicate spindle microtubule staining. **(B)** Antibodies specific for the inner-centromere marker, INCENP. Arrowheads indicate centromeres flanked by kinetochore-associated SENP1 and SENP2 signals. **(C)** Antibodies specific for the outer-kinetochore marker, Hec1. DNA was labeled with DAPI. Cells were imaged by immunofluorescence microscopy. Bar = 10 μm.

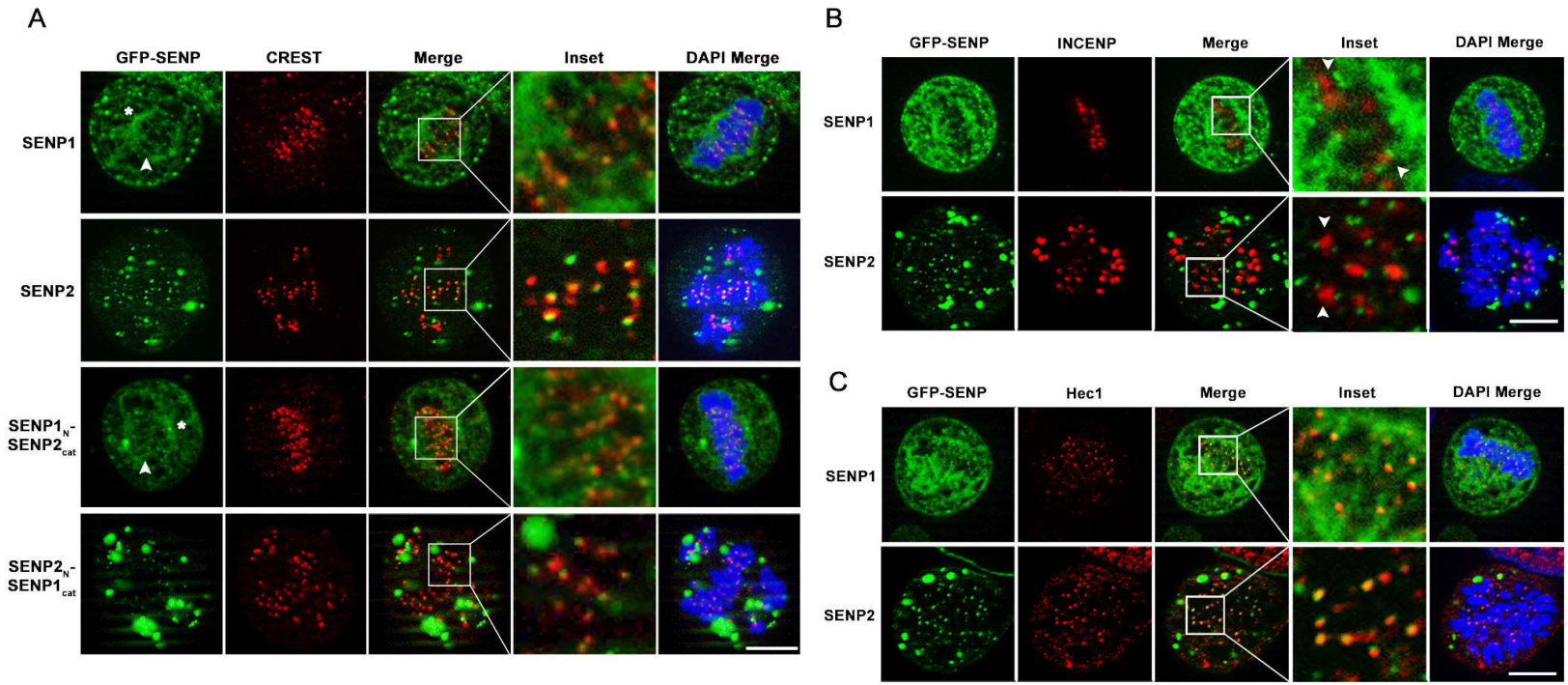

A
GFP-SENP
CREST
Merge
Inset
DAPI Merge
SENP1
SENP2
SENP1N-SENP2cat
SENP2N-SENP1cat
B
GFP-SENP
INCENP
Merge
Inset
DAPI Merge
SENP1
SENP2
C
GFP-SENP
Hec1
Merge
Inset
DAPI Merge
SENP1
SENP2

Figure 3-4. SENP1 and SENP2 interact with Nup107, but differentially associate with karyopherin α3. 293T cells were transfected with constructs coding for GFP, GFP-SENP1, or GFP-SENP2. Protein complexes were immunopurified, using GFP-specific antibodies, from lysates prepared from cells grown asynchronously or synchronized in mitosis by overnight incubation in the presence of nocodazole. Fractions of starting cell lysates (Input) and immunopurified protein complexes (IP) were analyzed by immunoblot analysis with antibodies specific for GFP, Nup107, karyopherin α3, tubulin, or phosphorylated histone H3. Astericks denotes a contaminating band co-migrating with GFP.

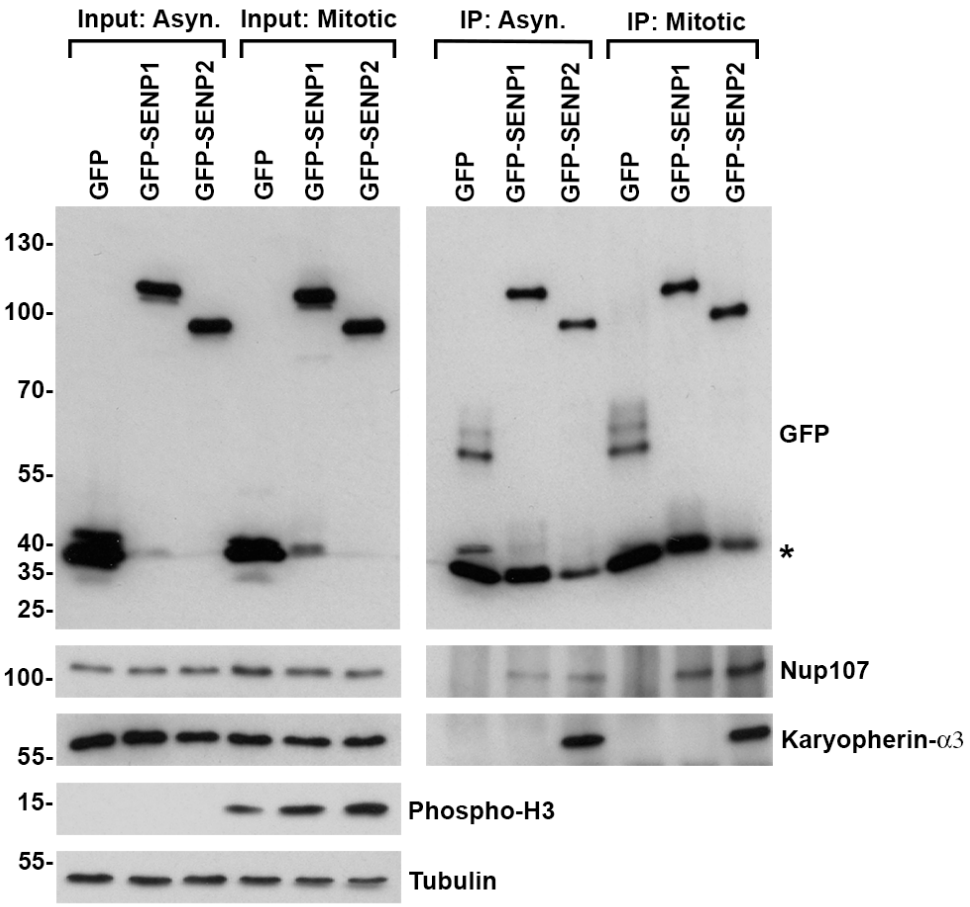

Input: Asyn.
Input: Mitotic
IP: Asyn.
IP: Mitotic
GFP
GFP-SENP1
GFP-SENP2
130-
100-
70-
55-
40-
35-
25-
GFP
*
100-
Nup107
55-
Karyopherin-α3
15-
Phospho-H3
55-
Tubulin

Figure 3-5. N-terminal targeting elements in SENP2 specify mitotic arrest phenotypes. **(A)** Schematic diagram of SENP2 and targeting domain mutants. Abbreviations: NLS, nuclear localization signal; BD, binding domain; CAT, catalytic domain. **(B)** HeLa cells were co-transfected with constructs coding for Myc-tagged SUMO-2 and wild type GFP-SENP2, the indicated GFP-tagged SENP2 mutants, or empty vector as control (Mock). Cell lysates were analyzed by immunoblot analysis with antibodies specific for Myc, GFP or tubulin. **(C)** HeLa cells were transfected with constructs coding for wild type GFP-SENP2, the indicated GFP-tagged SENP2 mutants, or empty vector (Mock). The fraction of transfected cells in mitosis was determined by fluorescence microscopy 48 hrs following transfection. **(D)** HeLa cells were transfected with constructs coding for wild type GFP-SENP2 or the indicated GFP-tagged SENP2 mutants. Cells were permeabilized, fixed and stained with Hec1 specific antibodies and analyzed by immunofluorescence microscopy. DNA was labeled with DAPI. Bar = 10 μm. Where indicated, error bars represent standard deviations from three independent experiments.

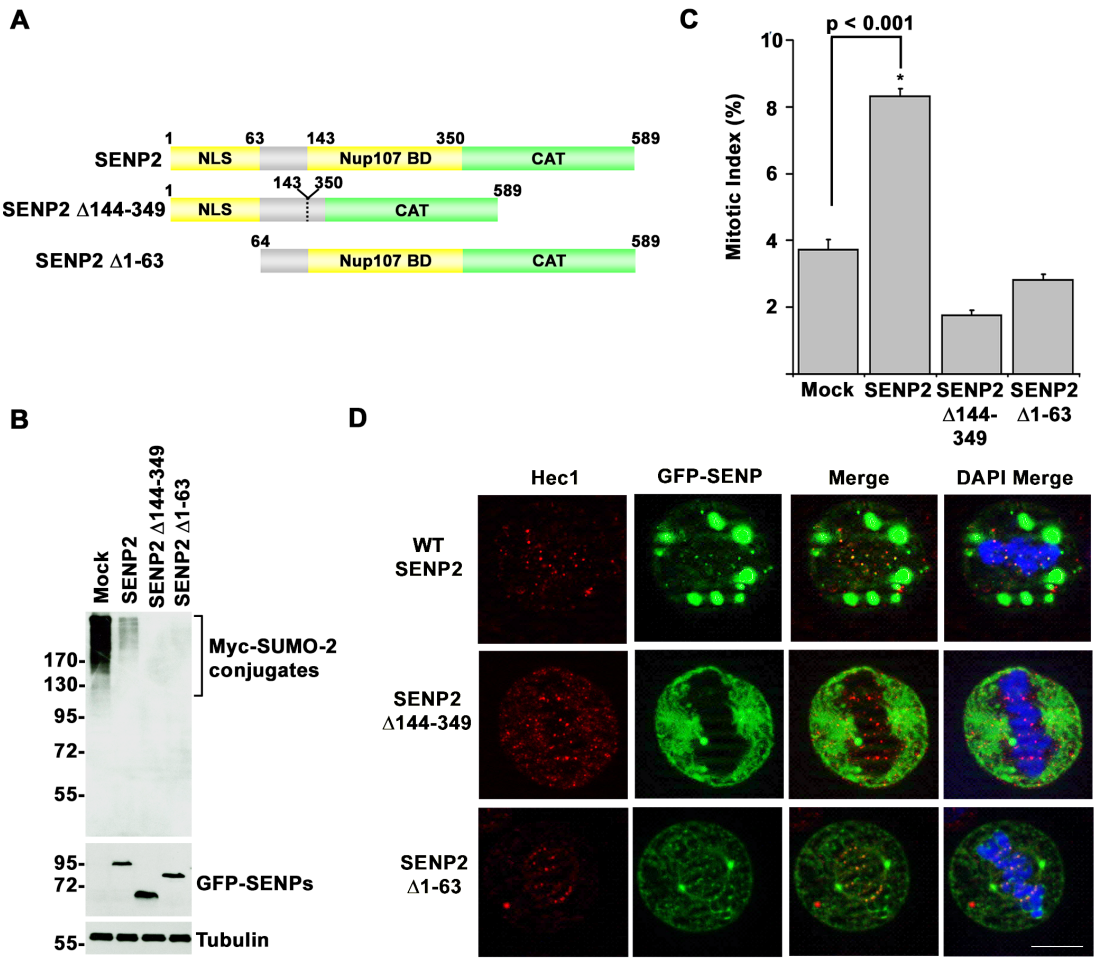

A
SENP2
1
63
143
350
589
NLS
Nup107 BD
CAT
SENP2 Δ144-349
SENP2 Δ1-63
64
B
Mock
SENP2
SENP2 Δ144-349
SENP2 Δ1-63
170-
130-
95-
72-
55-
Myc-SUMO-2 conjugates
GFP-SENPs
Tubulin
C
p < 0.001
Mitotic Index (%)
10
8
6
4
2
Mock
SENP2
SENP2 Δ144-349
SENP2 Δ1-63
D
Hec1
GFP-SENP
Merge
DAPI Merge
WT SENP2
SENP2 Δ144-349
SENP2 Δ1-63

Figure 3-6. Colocalization of karyopherin α3 and SENP2 is observed in interphase cells but is not detectable in mitotic cells. HeLa cells were transfected with constructs coding for GFP-SENP2, fixed and permeabilized 48 hours post-transfection. Cells were stained with karyopherin α3aryopherin st-transfection. Cells were stained with karyopherin karyopherinpermeabilized 48 hoar = 10 μm.

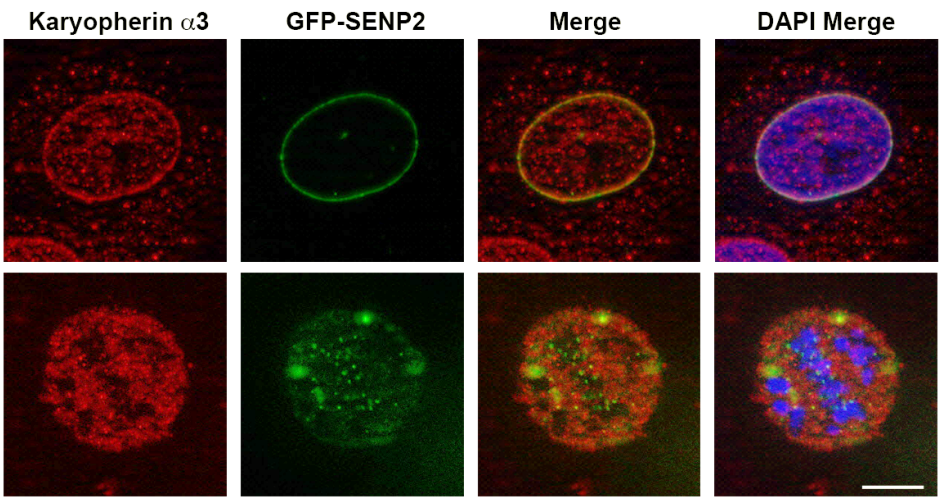

Karyopherin α3
GFP-SENP2
Merge
DAPI Merge

Figure 3-7. SENP1 induces mitotic arrest when artificially tethered to kinetochores. **(A)** Schematic diagram of HA-tagged SENP1-FKBP and FLAG-tagged FRB-Nuf2 fusion proteins. **(B)** HeLa cells were transfected with empty vector, or constructs coding for FRB-Nuf2, wild type SENP1-FKBP, or catalytically inactive SENP1-FKBP (C603A), as indicated. Cells were cultured in the presence (+) or absence (-) of AP21967 heterodimerizer. FLAG-tagged Nuf2 was immunopurified from cell lysates and immunoblot analysis was performed on starting cell lysates (Input) and immunopurified complexes using FLAG, HA and tubulin specific antibodies (IP). Asterisk denotes antibody heavy chain. **(C)** HeLa cells were co-transfected with constructs coding for HA-tagged SENP1-FKBP and FLAG-tagged FRB-Nuf2. Cells were cultured in the absence or presence of AP21967 heterodimerizer and analyzed by immunofluorescence microscopy using HA and FLAG specific antibodies. DNA was labeled with DAPI. Bar equals 10 μm. **(D)** Cells were transfected with constructs coding for the indicated fusion proteins and cultured in the absence or presence of AP21967 heterodimerizer. Mitotic indexes were determined by fluorescence microscopy. Error bars denote standard deviations from three independent experiments. **(E)** Illustration of mitotic chromosomes observed in cells co-expressing FRB-Nuf2 and SENP1-FKBP and cultured in the absence or presence of AP21967 heterodimerizer.

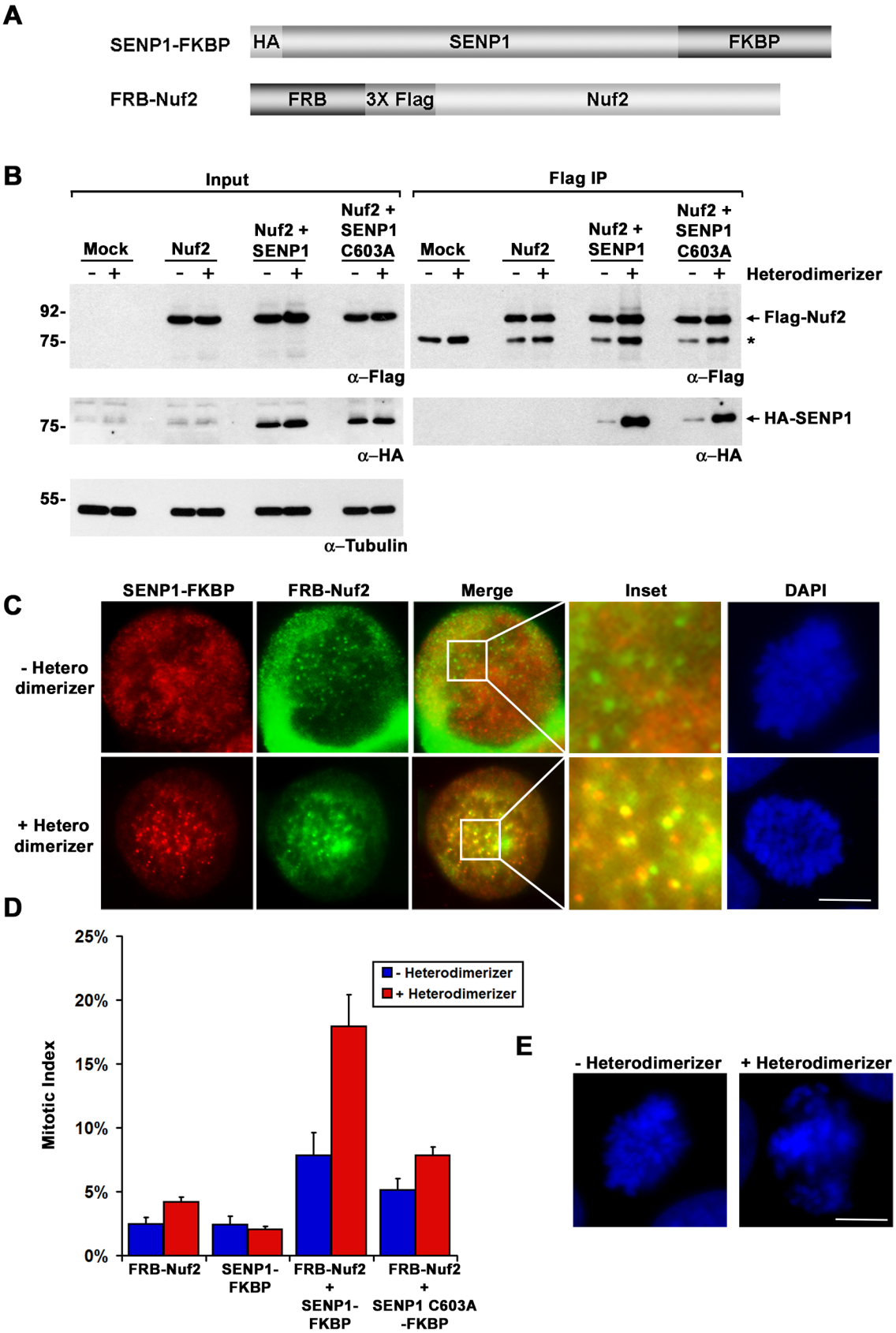

A
SENP1-FKBP
HA
SENP1
FKBP
FRB-Nuf2
FRB
3X Flag
Nuf2
B
Input
Flag IP
Mock
Nuf2
Nuf2 + SENP1
Nuf2 + SENP1 C603A
- +
Heterodimerizer
92-
75-
← Flag-Nuf2
*
α–Flag
75-
← HA-SENP1
α–HA
55-
α–Tubulin
C
SENP1-FKBP
FRB-Nuf2
Merge
Inset
DAPI
- Hetero dimerizer
+ Hetero dimerizer
D
25%
20%
15%
10%
5%
0%
Mitotic Index
- Heterodimerizer
+ Heterodimerizer
FRB-Nuf2
SENP1-FKBP
FRB-Nuf2 + SENP1-FKBP
FRB-Nuf2 + SENP1 C603A -FKBP
E
- Heterodimerizer
+ Heterodimerizer

Figure 3-8. Immunofluorescence microscopy analysis of endogenous SENP1 and SENP2. HeLa cells were fixed, permeabilized, stained, and analyzed by immunofluorescence microscopy. DNA was labeled with DAPI. Cells were co-labeled with: **(A)** SENP1 or SENP2 specific antibodies and mAb 414 to detect nuclear pore complexes in interphase. **(B)** SENP1 or SENP2 specific antibodies and Hec1 specific antibodies in mitosis. **(C)** SENP1 and tubulin specific antibodies in mitosis. Bar equals 10 μm.

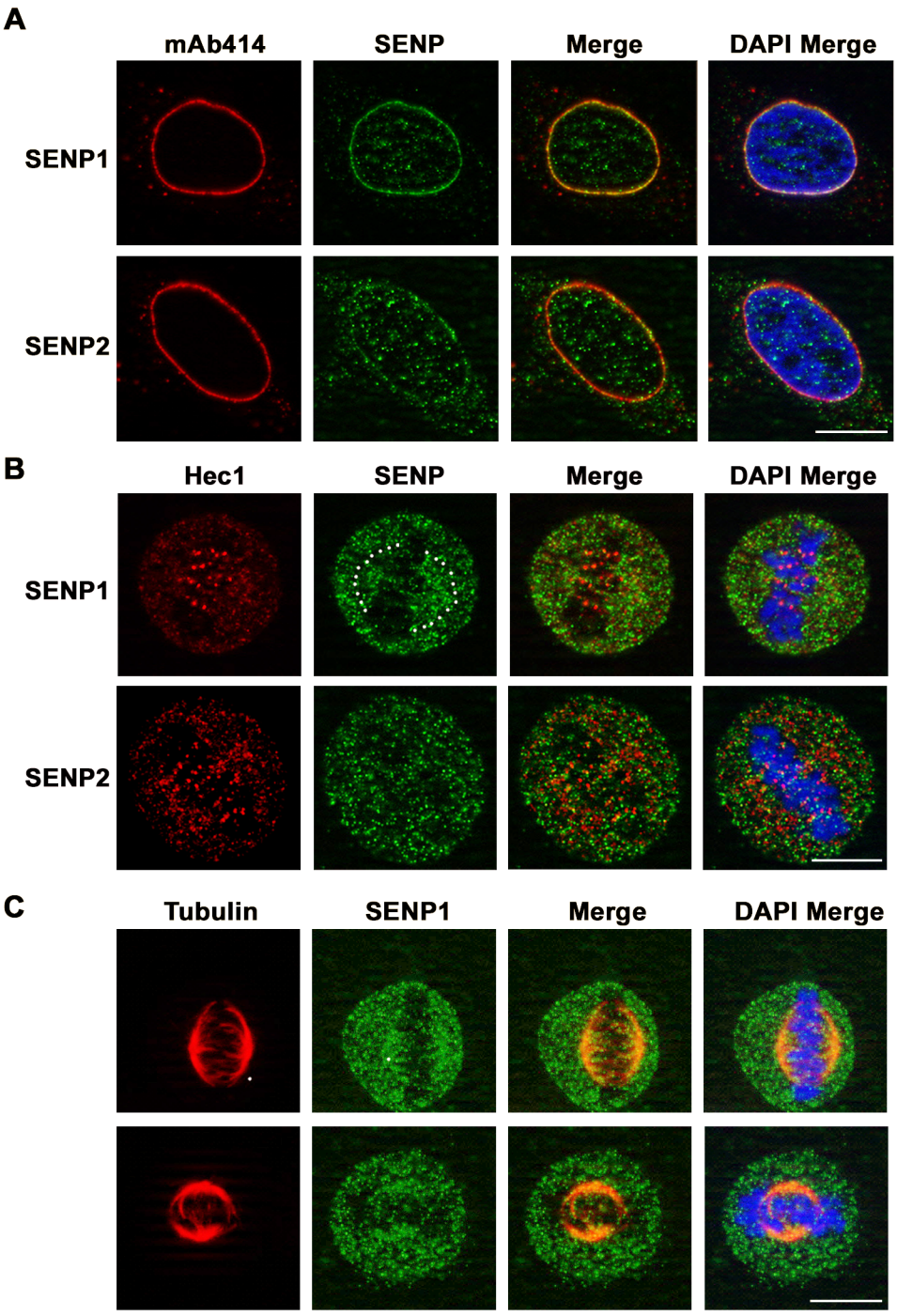
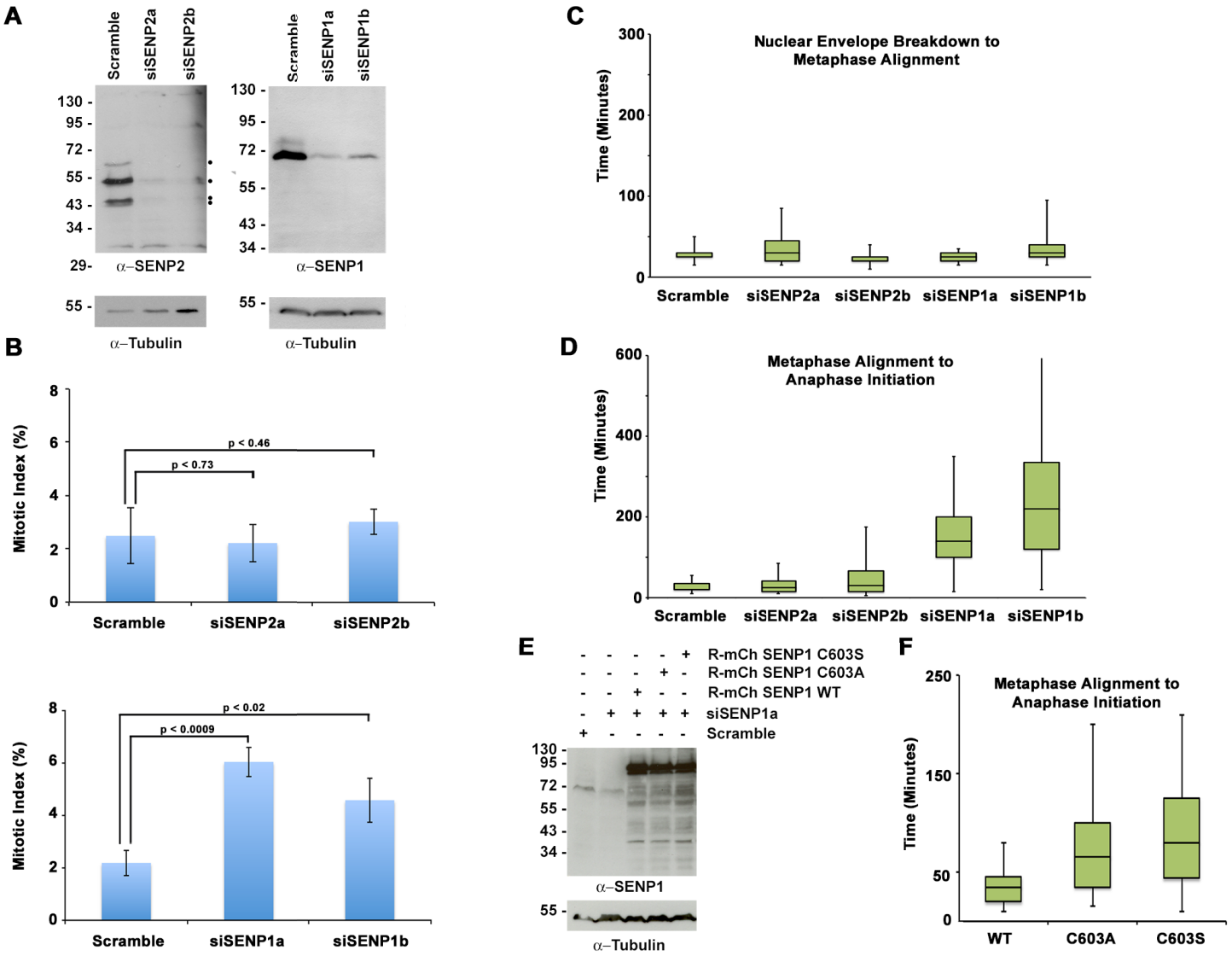

A
mAb414
SENP
Merge
DAPI Merge
SENP1
SENP2
B
Hec1
SENP
Merge
DAPI Merge
SENP1
SENP2
C
Tubulin
SENP1
Merge
DAPI Merge

Figure 3-9. Analysis of endogenous SENP1 and SENP2 by siRNA knockdown. (A and B) HeLa cells were transfected with a control scramble siRNA, two independent SENP-2 specific siRNAs, or two independent SENP1 specific siRNAs. **(A)** Cell lysates were analyzed by immunoblot analysis with SENP1 or SENP2 specific antibodies and anti-tubulin antibodies as indicated. Dots indicate multiple SENP2 isoforms. **(B)** Mitotic indices were determined by fluorescence microscopy analysis of DAPI stained cells 48 hours after transfection. Error bars equal the standard deviations from three independent experiments. **(C and D)** YFP-H2B expressing HeLa cells were transfected with control scramble, SENP1 or SENP2 siRNAs and imaged by live-cell fluorescence microscopy starting 48 hours after transfection. **(C)** Time from nuclear envelope breakdown to metaphase alignment and **(D)** metaphase alignment to anaphase onset was quantified for ≥65 cells from at least three independent experiments for each condition. Outliers, as defined as being 1.5 x the interquartile range above or below the dataset minimum or maximum, are not shown. **(E and F)** YFP-H2B expressing HeLa cells were co-transfected with control scramble or SENP1a siRNA and siRNA resistant constructs of mCherry-SENP1 WT or the catalytic mutants C603A and C603S. **(E)** Cells lysates were analyzed by immunoblot analysis with SENP1 or tubulin specific antibodies. **(F)** Time from metaphase alignment to anaphase onset was measured by live-cell fluorescence microscopy starting 48 hours after transfection and quantified for ≥35 transfected cells from three independent experiments for each condition. Outliers, as defined as being 1.5 x the interquartile range above or below the dataset minimum or maximum, are not shown.


A
Scramble
siSENP2a
siSENP2b
130 -
95 -
72 -
55 -
43 -
34 -
29-
α–SENP2
55 -
α–Tubulin
Scramble
siSENP1a
siSENP1b
130 -
95 -
72 -
55 -
43 -
34 -
α–SENP1
55 -
α–Tubulin
B
Mitotic Index (%)
8
6
4
2
0
p < 0.46
p < 0.73
Scramble
siSENP2a
siSENP2b
Mitotic Index (%)
8
6
4
2
0
p < 0.02
p < 0.0009
Scramble
siSENP1a
siSENP1b
C
Nuclear Envelope Breakdown to Metaphase Alignment
Time (Minutes)
300
200
100
0
Scramble
siSENP2a
siSENP2b
siSENP1a
siSENP1b
D
Metaphase Alignment to Anaphase Initiation
Time (Minutes)
600
400
200
0
Scramble
siSENP2a
siSENP2b
siSENP1a
siSENP1b
E
- - - - + R-mCh SENP1 C603S
- - - + - R-mCh SENP1 C603A
- - + - - R-mCh SENP1 WT
- + + + + siSENP1a
+ - - - - Scramble
130 -
95 -
72 -
55 -
43 -
34 -
α–SENP1
55 -
α–Tubulin
F
Metaphase Alignment to Anaphase Initiation
Time (Minutes)
250
150
50
0
WT
C603A
C603S

CHAPTER 4

SUMO: A MULTI-FACETED MODIFIED OF CHROMATIN STRUCTURE AND FUNCTION

ABSTRACT

A major challenge in nuclear organization is the packaging of DNA into dynamic chromatin structures that can respond to changes in the transcriptional requirements of the cell. Posttranslational protein modifications, of histones and other chromatin-associated factors, are essential regulators of chromatin dynamics. In this chapter, we summarize studies demonstrating that posttranslational modification of proteins by small ubiquitin-related modifiers (SUMOs) regulates chromatin structure and function at multiple levels and through a variety of mechanisms to influence gene expression and maintain genome integrity.

MAIN TEXT

Introduction

The discovery of the nucleosome, the iconic "beads on a string," and finally the realization that there are higher order chromatin packing structures have made it clear that DNA is intricately organized. Since this time, significant progress has been made in identifying the proteins responsible for higher order DNA packaging and in understanding how regulation of these proteins affects chromatin structure. A major theme that has emerged is the important role of posttranslational protein modifications in modulating the functional accessibility of DNA. Of particular interest, recent global proteomic and genetic studies have linked modification by the small ubiquitin-related modifier (SUMO) to many processes involving chromatin, including transcriptional activation and repression, DNA replication and repair, as well as chromosome segregation (1,2). Here, we review the current knowledge of how SUMO modification (sumoylation) of chromatin-associated proteins regulates chromatin structure and function and thereby controls these essential cellular processes.

After introducing the sumoylation pathway and general connections between SUMO and chromatin, we will discuss the complex role of sumoylation in both euchromatin and heterochromatin environments. First, the multiple mechanisms by which sumoylation modulates gene expression through effects on DNA methylation, histones and transcriptional regulators will be reviewed. Subsequently, the functional role of sumoylation in repetitive DNA structures, including rDNA, telomeres, and centromeres will be discussed. We will highlight the unique functions of sumoylation

within each of these domains as well as its common role as a protector of genomic integrity.

Several emerging themes will be reiterated throughout the review. First, that sumoylation often functions as a signal to facilitate protein-protein interactions on chromatin. These interactions may be simple hetero-dimeric associations, but can also involve assembly of very large multi-protein complexes. Second, that sumoylation also specifies multiple other fates, including effects on enzyme activity and changes in protein sub-cellular localization. And lastly, that although in many cases sumoylation is linked to heterochromatin and gene inactivation, a growing number of studies indicate that sumoylation also plays important roles in enhancing chromatin accessibility and gene activation. Thus, the effects of sumoylation are dichotomous and often context dependent.

SUMO Modification and Function

Mechanistically, sumoylation occurs through an enzyme cascade very similar to ubiquitylation (Figure 4-1A). The SUMO paralogs are synthesized as precursor proteins that are cleaved by a family of SUMO isopeptidases referred to as SENPs (3). Mature SUMO is subsequently activated by a heterodimeric E1 activating enzyme (Aos1/Uba2), transferred to an E2 conjugating enzyme (Ubc9), and finally transferred to lysine residues in target proteins. This last step may be facilitated by the action of E3 ligases, which in addition to enhancing rates of sumoylation, are also believed to contribute to specificity (4,5). Substrate specificity in the sumoylation pathway, however, still remains poorly understood as only a single E2 enzyme and relatively few E3 ligases have been

identified. Sumoylation is, however, highly dynamic and can be reversed by the action of desumoylating enzymes. In vertebrates, these isopeptidases include a family of six SENPs defined by a conserved cysteine protease domain, distinct-subcellular localizations and non-redundant functions (Mukhopadhyay and Dasso, 2007). In addition, several unique desumoylating enzymes have more recently been identified, including the metalloprotease Wss1, the PPPDE–domain containing proteins DeSI-1 and DeSI-2, and the ubiquitin-specific protease-like protein 1 (USPL1) ((3,6-8). Sumoylation of individual proteins is likely to be regulated by a fine-tuned balance between conjugation and deconjugation (5). Consistent with this, and as outlined below, both SUMO conjugating and deconjugating enzymes are important effectors of chromatin structure.

Sumoylation of proteins can affect protein stability, enzymatic activity, alter localization, or mediate novel protein-protein interactions with other proteins containing SUMO-interacting motifs (SIMs) (Figure 4-1B) (9,10). In many instances, sumoylation may play a role in facilitating the assembly of large multi-protein complexes between proteins that are either covalently modified by SUMO and/or contain SIMs, as exemplified by PML nuclear bodies. In these sub-nuclear structures, SUMO acts as a scaffold to mediate interactions between the PML protein and other associated factors (11,12). Although multiple effects of sumoylation on proteins have been discovered, the ability of SUMO to promote the assembly of multi-protein complexes is an especially prominent theme.

The diverse effects of sumoylation may be explained in part through the generation of functionally distinct signals. Although invertebrates express only a single

SUMO, vertebrates express four paralogs (SUMO-1, SUMO-2, SUMO-3 and SUMO-4), each with the potential to act as unique signals by interacting with distinct downstream factors (9). SUMO-2 and SUMO-3 share ~97% identity with each other and likely represent redundant signals and are thus referred to as SUMO-2/3. However, they share only ~50% identity with SUMO-1 (5). SUMO-4 shares ~86% identity with SUMO-2/3, but questions exist about its ability to be conjugated to other proteins (6,13). The ability of SUMOs to form polymeric chains provides an additional opportunity for signal diversification (Figure 4-1B) (9). Currently the best-studied functional role for polymeric SUMO chains involves their recognition by SUMO-targeted ubiquitin E3 ligases containing tandem SIMs (14). Other functional distinctions between paralogs and polymers remain to be fully understood. Finally, the diverse effects of sumoylation can also be explained through intersections with other posttranslational modification pathways (Figure 4-1B). For example, both phosphorylation and acetylation affect interactions between SUMO and downstream SUMO-binding proteins (15,16).

General connections between sumoylation, chromatin and transcription

Associations between sumoylation and chromatin structure have been well documented through numerous immunofluorescence microscopy studies. All three SUMO paralogs, for example, are detected in the heterochromatin XY bodies of rat pachytene spermatocytes (17-20), and SUMO-1 is associated with long stretches of constitutive heterochromatin in human spermatocytes (21). In mitotic cells, SUMO-2/3 has been observed at the inner centromere of chromosomes and also along the length of chromosome arms as cells progress from metaphase through telophase (22-24).

Associations between SUMO and mitotic chromosomes are also detected in *S. cerevisiae* (25) and in *D. melanogaster* using polytene chromosome spreads (26), suggesting that sumoylation of chromatin-associated proteins has a conserved and fundamentally important function.

Associations between SUMO and chromatin are further supported by biochemical studies, including chromatin immunoprecipitation experiments (ChIP). In *S. pombe*, for example, ChIP experiments revealed that the SUMO E2 conjugating enzyme Ubc9 is chromatin bound and specifically enriched in regions of heterochromatin (27). Similarly, fractionation of *X. laevis* egg extracts demonstrated interactions between PIAS E3 ligases and chromatin (23). Surprisingly, a comprehensive genome-wide ChIP analysis to detect the precise association of SUMO or SUMO-modified proteins with chromatin has not yet been reported. However, more targeted studies link SUMO or SUMO pathway enzymes to distinct chromatin domains, including pericentric heterochromatin, PcG bodies, the nucleolus, telomeres, and centromeres, as reviewed in detail below.

Studies related to the involvement of sumoylation in controlling transcription regulation provide the strongest evidence for functional links between SUMO and chromatin. Genetic approaches have revealed a general causal relationship between sumoylation and gene repression. Inducing hyper-sumoylation by targeting SUMO and/or Ubc9 to specific gene promoters primarily induces gene repression (28,29). Consistently, inducing hypo-sumoylation by overexpressing SUMO isopeptidases or by depleting cells of Ubc9 or SUMO enhances ectopic gene expression (30-32). These effects are mediated at multiple levels, including direct effects on transcription factor activities (33). Transcription factors and co-regulators make up one of the most abundant

classes of SUMO-modified proteins. Although clearly able to mediate transcriptional repression, sumoylation is not simply a negative regulator of transcription. The dichotomous role of SUMO in gene regulation is demonstrated by the observations that sumoylation of certain transcription factors, including Ikaros, enhance their transcriptional activity (Figure 4-2E) (34).

Studies in yeast also provide a striking example of the complexities of sumoylation as both an activator and repressor of transcription. ChIP analysis in *S. cerevisiae* reveals the presence of sumoylated proteins at the promoters of constitutively active genes and the recruitment of Ubc9 and SUMO to promoters of inducible genes in response to activation (35). Surprisingly, sumoylation is not only required for optimal transcriptional activation of constitutive genes but also for repression and timely inactivation of inducible genes. At constitutively active genes, sumoylation enhances transcription by promoting RNA polymerase II recruitment (Figure 4-2F). However, at inducible promoters sumoylation functions downstream of transcription initiation. Specifically, sumoylation of the transcription factor Gcn4 promotes its removal from promoters and its degradation, thereby limiting transcription reinitiation (35,36).

Another elegant example of the subtle and complex effects of sumoylation on transcription is illustrated by phenotypes in mice expressing a mutant form of the SF-1 transcription factor that cannot be sumoylated. Although studies in cultured cells indicate that sumoylation negatively regulates SF-1 transcriptional activity, mice expressing non-sumoylatable SF-1 fail to phenocopy a constitutively active SF-1 (37,38). Thus, sumoylation does not function as a simple on-off switch, but rather enhances the

functional diversity of SF-1, adding a layer of regulation for fine-tuning gene expression during development.

The utility of sumoylation as a mechanism to fine tune transcription can be explained in part on its broad effects on chromatin modifications and structure. This is illustrated by studies of a well-characterized SUMO-1 modified transcription factor, Sp3. In cells expressing specific mutant isoforms of Sp3 that cannot be sumoylated, transcription activation and chromatin modifications at Sp3-targeted promoters are dramatically different from those observed at the same promoters in cells expressing wild type Sp3. For instance, levels of both DNA and histone methylation are reduced at promoters in cells expressing mutant Sp3 and, concomitantly, levels of histone methyltransferases, heterochromatin protein 1 (HP1), and two ATP-dependent chromatin remodelers are also reduced (39-42). These findings provide a relatively simple view of how sumoylation of just one transcription factor exerts multiple effects, some direct and others indirect, to alter chromatin structure. However, even in the case of Sp3, the situation is not so simple, as evidenced through additional studies demonstrating that the effects of sumoylation are unique for different Sp3 isoforms and for different gene promoters (43,44). An emerging view is that sumoylation sits at the intersection of multiple pathways, affecting the activities of not only transcription factors, but also other chromatin-associated proteins and chromatin modifying enzymes. Thus, effects of sumoylation on gene expression and chromatin structure represent collective effects on multiple, context-dependent, levels (Figure 4-2). How sumoylation affects gene expression at the level of chromatin structure and accessibility, and within the context of distinct genomic subdomains, is the focus of the following sections.

DNA Methylation

DNA methylation of CpG dinucleotides restricts DNA accessibility by two mechanisms. Methylation either blocks the binding of sequence-specific DNA binding proteins and/or recruits chromatin modifying complexes that promote a restrictive chromatin structure (45). Multiple lines of evidence indicate that sumoylation plays important roles in regulating CpG methylation and demethylation, as well as the assembly and functions of downstream complexes recruited to methylated DNA.

First, sumoylation of DNA methyltransferases (Dnmts) may alter their enzymatic activity (Figure 4-2A). This has been demonstrated most clearly for the maintenance methyltransferase, Dnmt1, whose SUMO-1 modification increases its activity toward S phase hemi-methylated DNA substrates *in vitro* (46). In addition, the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b are both sumoylated *in vivo*, although the functional consequences of their modifications remain to be fully elucidated (47-49). Strikingly, nearly all of Dnmt3a is sumoylated in cells overexpressing SUMO-1, an effect that correlates with a disruption of Dnmt3a interactions with histone deacetylases 1 and 2 (HDAC1 and -2) and a loss of Dnmt3a-mediated repression (49). Further studies are needed to determine whether these effects are strictly related to Dnmt3a sumoylation.

In addition to regulating DNA methylation, sumoylation also promotes DNA demethylation through mechanisms mediated by RNF4, a ubiquitin E3 ligase that specifically recognizes and ubiquitylates sumoylated proteins (Figure 4-2D) (14,50). RNF4 deficiency is embryonic lethal in mice. $RNF4^{-/-}$ mouse embryonic fibroblasts, however, are viable but exhibit hypermethylation of genomic DNA. Conversely, overexpression of wild type RNF4, but not SUMO-binding or ubiquitin ligase mutants,

results in global DNA demethylation (50). Thus, SUMO and ubiquitylation are required for RNF4-mediated DNA demethylation, although the precise mechanisms of action remain unclear. Intriguingly, one favored model for DNA demethylation is based on deamination of methylcytosines to create T:G mismatches that are repaired by thymidine DNA glycosylase (TDG) and base-excision repair (BER) (51). TDG is known to interact with RNF4 (50) and sumoylation has been proposed to play an important role in regulating TDG by enhancing its enzymatic turnover (52,53). Thus, ubiquitylation of sumoylated TDG or other interacting proteins could produce a signal required for DNA demethylation and possibly BER in general.

Sumoylation also functions downstream of DNA methylation, affecting the assembly of methyl-CpG binding domain (MBD) proteins and other factors with methylated DNA (Figure 4-2B) (54). SUMO-1 and SUMO-2/3 both localize to heterochromatin domains enriched in MBD1, as well as heterochromatin proteins HP1 and MCAF1 (55). Formation of these heterochromatin domains is SUMO-dependent, as knockdown of either SUMO-1 or SUMO-2/3 disrupts the co-localization of HP1 and MCAF1 with MBD1-containing foci (55). Intriguingly, MBD1 and HP1 are both sumoylated, whereas MCAF1 binds all three SUMO paralogs (55-57). Thus, it is tempting to speculate that MBD1-containing heterochromatin domains are organized around covalent and non-covalent SUMO interactions in a fashion similar to PML nuclear bodies (11). In contrast with these repressive functions, however, sumoylation of MBD1 also interferes with its interactions with the histone methyltransferase SETDB1 and might thereby limit gene inactivation (56). Therefore, sumoylation underlies multiple mechanisms for fine-tuning the functional properties of methylated DNA

through effects both positive and negative, again demonstrating the dichotomous effects of SUMO on gene expression.

Histones and HDACs

Posttranslational modification of histones also represents a central mechanism for controlling chromatin structure and gene expression, and not surprisingly, histones are sumoylated. All four histones as well as the H2A.Z variant are sumoylated in *S. cerevisiae* (58,59), whereas only H4 has been shown to be modified in mammalian cells (29). The functional significance of histone sumoylation is surprisingly not well understood. ChIP experiments involving exogenously expressed SUMO-histone fusion proteins in yeast reveals enrichment at subtelomeric regions, an area of the genome where SUMO is generally thought to antagonize transcriptional repression (58,60,61). In contrast, expression of SUMO-histone fusion proteins represses transcriptional reporters in both mammalian cells and in yeast at least in part through recruitment of histone deacetylases (HDACs) and HP1 (29,58). Such findings suggest that histone sumoylation functions as a signal to recruit proteins to chromatin (Figure 4-2B). Consistent with this general concept, recruitment of the transcription corepressor complex, LSD1/CoREST1/HDAC, to chromatin is dependent on a SIM in CoREST1 (30). Whether CoREST1 recognizes sumoylated histones and/or other sumoylated factors, however, remains to be determined.

In addition to histones, multiple studies have identified HDACs as another important effector of SUMO-mediated transcriptional repression. Most simply, HDACs themselves are sumoylated (Figure 4-2B). Sumoylation of HDAC1 and HDAC4 is

required for the full transcriptional repression activities at defined promoters (62-64). Whether sumoylation directly affects HDAC activity or acts as a signal for the recruitment of other chromatin repressors, however, is a question that remains to be fully addressed. In addition to being directly modified, HDACs are also recruited to gene promoters in response to sumoylation of other factors, including transcription factors and cofactors such as Elk-1 and p300 (65-67). These findings suggest that HDAC recruitment may be mediated through non-covalent interactions with SUMO, a suggestion that has been confirmed for at least HDAC1 which contains a functionally important SIM (68). A third level of association between HDACs and sumoylation has been made based on the observations that HDACs 4, 5, and 7 appear to function as SUMO E3 ligases for certain substrates (69-71). These findings are based largely on effects of HDAC overexpression, where an alternative mechanism for enhanced sumoylation might involve substrate binding and protection from isopeptidases. In either case, HDAC interaction would provide a feed-forward mechanism for enhancing sumoylation-mediated histone deacetylation and repression.

PcG Bodies

Polycomb group (PcG) bodies are subnuclear structures that function as small hubs of transcriptional repression. To facilitate repression, PcG bodies cluster distant DNA promoter elements and recruit chromatin remodeling complexes called polycomb repressive complexes (72). Given its involvement in repression and organizing large protein complexes, it is not surprising that SUMO localizes to PcG bodies (Figure 4-2C) (73). In addition to SUMO, Ubc9, the SUMO isopeptidase SENP2, and the SUMO E3

ligase Cbx4/Pc2 all localize to PcG bodies (73-75). Because Pc2 stimulates the sumoylation of many repressive proteins, including Dnmt3a, CTCF, and components of the polycomb repressive complex 2, it is attractive to speculate that sumoylation regulates the dynamic recruitment and assembly of these proteins within PcG bodies in a fashion similar to PML nuclear bodies (11,48,76,77).

Consistent with essential functions in PcG body-mediated repression, two independent studies have demonstrated links between sumoylation and expression of PcG-body regulated genes. In *C. elegans*, depletion of SUMO, E1 or E2 conjugating enzymes results in ectopic expression of Hox genes normally controlled by PcG body recruitment (78). The appropriate repression of Hox genes is dependent at least in part on sumoylation of the PcG protein SOP-2, which is required for the association of SOP-2 with PcG bodies (78). SUMO-dependent assembly of PcG bodies is also conserved in mammalian cells and is also critical for normal gene expression during embryonic development. In particular, assembly of the polycomb repressive complex 1 (PRC1) at the promoters of genes important for normal heart development is misregulated in mice deficient in the SENP2 isopeptidase (75). This misregulation is due in part to hypersumoylation of the Cbx4/Pc2 SUMO E3 ligase and enhanced assembly of PRC1 complexes on the promoters of PcG target genes. These findings illustrate the important balance between SUMO conjugating enzymes and isopeptidases, which is a common theme in ubiquitylation (79). Further studies are required to understand how the activities of Cbx/Pc2 and SENP2 are normally regulated to affect proper PcG body function.

Finally, in another example of the dichotomous effects of sumoylation, assembly of the PcG protein Sex Comb on Midleg (Scm) into repressor complexes in *D.*

melanogaster appears to be negatively regulated by its SUMO modification (80). Whether sumoylation has universally opposing effects on PcG body formation in *D. melanogaster* compared to other organisms remains to be determined. An alternative and more appealing scenario is that sumoylation both positively and negatively affects PcG body assembly, with the ultimate effects on individual protein recruitment and gene expression being influenced by multiple, context-dependent factors and interactions.

Chromatin insulators

Sumoylation also influences gene expression by affecting the activities of chromatin insulator complexes. This function was first revealed by studies in *D. melanogaster*, demonstrating that loss of the PIAS E3 ligase homolog results in ablation of heterochromatin-euchromatin barriers and normal polytene chromosome banding patterns (81). Consistent with a role in regulating insulator functions, SUMO was subsequently localized to insulator bodies in *D. melanogaster* and two of the major protein components, Mod(mdg4)2.2/67.2 and CP190, were found to be sumoylated (82,83). However, the function of SUMO in organizing and regulating the function of insulators is still unclear. Enhancing sumoylation by Ubc9 overexpression leads to dispersal of insulator bodies, suggesting that sumoylation may negatively affect local and/or long-range interactions between insulator complexes (82). In contrast, SUMO depletion or expression of a Mod(Mdg4)2.2/67.2 mutant that cannot be sumoylated inhibits insulator body formation, arguing for a positive role in insulator assembly (83). Such opposing findings indicate that insulator assembly and/or maintenance may rely on a finely tuned balance of sumoylation and desumoylation, as required for the association

or HP1α with pericentric DNA (84). Further analysis is needed to understand the function of SUMO in insulator activity in *Drosophila* and particularly in other species. CTCF, a well characterized vertebrate insulator protein, is sumoylated in human cells, but how sumoylation affects its insulating activities remains unknown (76).

The nucleolus

The nucleolus is a specialized sub-nuclear domain for ribosomal RNA (rRNA) gene expression and pre-ribosomal particle assembly (85). Studies in both vertebrates and yeast indicate that sumoylation plays important roles in the nucleolus, including regulation of rRNA processing and pre-ribosomal particle assembly (86-89). Consistent with this, SUMO-1 and SUMO-2/3 are detected within the nucleolus in vertebrate cells (22,90), as are the isopeptidases SENP3 and SENP5 (91). Sumoylation also appears to have important effects on nucleolar rDNA structure and function. Thus, hyposumoylation due to defects in the SUMO E3 ligase Mms21, a component of the Smc5/6 complex, lead to abnormal nucleolar morphology in *S. cerevisiae* (61). In addition, aberrant activation of silenced rDNA occurs in *S. cerevisiae* strains deficient in the Slx5/Slx8 SUMO-targeted ubiquitin E3 ligase (92). How sumoylation affects rDNA chromatin structure and silencing remains to be fully characterized. However, recent proteomic identification of the SUMO substrates within the nucleolus should enhance these efforts (93,94).

Because of the repetitive nature of rDNA genes, specialized SUMO-dependent DNA repair mechanisms exist to maintain stability of nucleolar rDNA loci (Figure 4-3E). DNA double-strand breaks within rDNA loci are repaired at extranucleolar sites in a

manner dependent on the Smc5/6 complex and sumoylation of Rad52. Specifically, yeast strains expressing Smc5/6 mutants or a Rad52 mutant that cannot be sumoylated form DNA repair foci within the nucleolus itself and these strains exhibit hyper-recombination within the rDNA locus (95,96). Whether Mms21-mediated sumoylation regulates DNA repair in other repetitive sequences by a similar mechanism remains to be determined, but the role of SUMO in general repetitive DNA maintenance is reviewed in detail below.

Telomeres

Telomeres are composed of DNA repeats coated by resident telomere-binding protein complexes, called shelterin complexes, that protect them from degradation and inappropriate recognition by DNA repair enzymes (97). Because telomere shortening jeopardizes genome integrity, maintenance of telomere length is a tightly controlled process. Telomere maintenance is controlled through multiple mechanisms, including recruitment of telomerase (the enzyme that catalyzes the addition of telomere DNA repeats), modulation of the heterochromatin environment of the subtelomeric regions, and modulation of the alternate lengthening of telomeres (ALT) pathway (97). In strains of *S. cerevisiae* and *S. pombe* defective in SUMO, Ubc9, or SUMO E3 ligases, telomeres are abnormally elongated, demonstrating a role for sumoylation in affecting one or more of these mechanisms (60,61,98-101).

The telomere elongation observed in yeast mutants defective in sumoylation is telomerase-dependent, suggesting that sumoylation normally limits the accessibility of telomeres to telomerase (Figure 4-3A) (101). To date, there is no demonstration that telomerase itself is regulated through sumoylation. Studies have shown that sumoylation

affects the activity of Cdc13, a single-stranded telomere binding protein and regulator of telomerase recruitment, by promoting Cdc13 association with the telomerase inhibitor, Stn1. Consequently, yeast strains expressing a Cdc13 mutant that cannot be sumoylated have lengthened telomeres, while shortened telomeres are observed in strains expressing a Cdc13-SUMO fusion (99). Furthermore, multiple components of the shelterin complex, which are known to limit telomerase recruitment to telomeres (102), are sumoylated (99,103-106). Sumoylation of one or more of these factors is likely to contribute to the full inhibitory effect of SUMO on telomere lengthening. However, the underlying molecular mechanisms remain unknown.

Sumoylation may also regulate telomere length by modulating the heterochromatin environment of the subtelomere (Figure 4-3B). Mutations in yeast that disrupt telomeric heterochromatin structure and silencing also cause shortened telomeres, indicating that heterochromatin proteins positively regulate telomere length (97). Because SUMO negatively regulates telomere length, this model would predict that sumoylation antagonizes silencing in the subtelomere. Remarkably, the data confirms this model despite the more general association of SUMO with enhanced repression. Reducing levels of sumoylation in yeast leads to increased telomeric silencing (60,61), while increasing sumoylation relieves telomeric silencing (92,107). The molecular mechanisms underlying these effects are not fully understood, but are likely to be complex. For instance, sumoylation is required for the clustering and anchoring of telomeres to the nuclear periphery, a process that stabilizes telomeric heterochromatin and limits telomerase activity (61,81,103).

Finally, sumoylation is involved in telomere maintenance through effects on the ALT pathway (4-3C). In mammalian cells that utilize ALT, knockdown of the SUMO E3 ligase Mms21 results in reduced telomere length and increased senescence. Although this may appear to contradict phenotypes observed in yeast, this effect is unique to ALT and is not observed if telomerase is introduced into cells (106). The requirement of sumoylation in ALT is explained in part because telomere elongation is dependent on assembly of subnuclear structures formed around telomeres called ALT-associated PML nuclear bodies (APBs). Similar to PML nuclear bodies, assembly of APBs is SUMO dependent. Artificially tethering SUMO or Mms21 to telomeric regions is sufficient to promote APB formation, while Mms21 knockdown limits APB formation (106,108). Thus, requirements for sumoylation in the ALT pathway are due at least in part to an essential role in APB formation.

Centromeres

Centromeres are specialized chromatin structures that form the foundation for kinetochores and are therefore essential for proper chromosome segregation during cell division (109). The gene encoding SUMO was first identified in yeast as a high copy suppressor of a mutant allele of the centromere-associated protein Mif2 (the vertebrate CENP-C homolog) (110), providing an early indication of a connection between sumoylation and centromeres. Since then, immunofluorescence microscopy has demonstrated that SUMO-2/3 localizes to centromeres on chromosomes formed in *Xenopus* egg extracts and on mammalian mitotic and meiotic chromosomes (17,20,22-24,111). Furthermore, various SUMO E3 ligases, including PIASy, PIAS3, and

Nup358/RanBP2, are also present at centromeres of mitotic chromosomes (81,112-114). Consistent with essential roles in regulating centromere and kinetochore function in mitosis, chromosome segregation defects occur when the SUMO pathway is either up or down regulated (24,25,81,113,115-117). Segregation, cohesion, and other roles for sumoylation during mitosis have been characterized and are reviewed in more detail elsewhere (118). Here, we focus more specifically on effects of sumoylation on centromeric heterochromatin.

In contrast to mitosis, few studies have addressed potential roles for sumoylation at centromeres or kinetochores during interphase. Of particular interest, it is not known whether SUMO remains associated with centromeres throughout the cell cycle or whether its association is specific to mitosis. A role for sumoylation in the maintenance of centromeric heterochromatin during interphase is, however, suggested by the findings that inhibiting sumoylation in both yeast and mammalian cells results in activation of genes within normally repressed centromeric regions (Figure 4-3D) (27,101,119). For example, *S. pombe* mutants lacking the Pli1 SUMO E3 ligase exhibit reduced silencing and enhanced conversion of genes inserted into core centromeric regions (60). How Pli1-dependent sumoylation normally restricts transcription and recombination within this centromeric region is not fully understood. Inhibition could be mediated through effects on transcription and recombination factors, or through more direct effects on the formation and/or maintenance of centromeric heterochromatin.

One mechanism by which centromeric chromatin structure is regulated by sumoylation is through recruitment of the heterochromatin factor HP1α (Figure 4-3D). Sumoylation of HP1α regulates its interactions with major α-satellite RNA transcripts

which in turn directs pericentric DNA targeting (57). Although sumoylation occurs within the hinge domain of HP1α thought to be involved in RNA binding, the exact molecular mechanisms underlying SUMO-dependent interactions with α-satellite RNAs remain to be determined. Intriguingly, further studies have revealed that depletion of SENP7, a SUMO protease that localizes to HP1-enriched pericentric domains, disrupts HP1α localization (84). This finding suggests that localization of HP1a is dependent on transient sumoylation of HP1α followed by desumoylation and may explain the common "SUMO enigma" (120), namely that steady state levels of sumoylated HP1α in the cell represent only a relatively minor fraction of total HP1α. A role for sumoylation in HP1 targeting and gene silencing has also been observed in *S. pombe,* where HP1 mutants that cannot be sumoylated are less efficiently recruited to heterochromatin domains and compromised in their ability to repress gene expression (27). Thus, sumoylation of HP1 represents an important and conserved regulatory point for controlling heterochromatin structure and gene expression at centromeres and other chromatin domains.

Maintenance of repetitive DNA

Repetitive DNA sequences, including those found at telomeres, centromeres and within the rDNA gene loci, represent especially fragile domains in the genome due to issues related to replication and recombination (121). The maintenance of these and other repetitive DNA domains is highly dependent on the activity of the cohesion-like complex, Smc5/6, and the associated SUMO E3 ligase, Mms21 (122). Similar to other SMC complexes, Smc5/6 activities are mediated at least in part through cohesion-related effects on higher-order chromatin structure, but also through the targeting of Mms21 to

appropriate DNA targets. Smc5/6 and Mms21 are essential for the maintenance of genome integrity, with mutants exhibiting gross chromosomal rearrangements and chromosome segregation defects. These defects are due in part to the incomplete resolution of replication-associated homologous recombination intermediates, particularly within the rDNA locus and at telomeres (Figure 4-3F) (96,122-125).

While the exact molecular targets and functions of sumoylation in the maintenance of heterochromatic repetitive DNA are not fully understood, a growing body of evidence indicates that replication through these domains requires DNA repair factors, including BRCA1 and Rad51 (126,127). Sumoylation is intimately linked to the control of these and a large number of other DNA repair factors (128-130), suggesting that Mms21-dependent sumoylation is required for proper resolution of DNA repair intermediates produced during replication. Consistent with this, recombination-dependent DNA repair intermediates accumulate during replication in Smc5/6, Ubc9 and Mms21-deficient cells (122,131).

Sumoylation also plays an essential role in regulating the resolution of DNA intermediates at centromeres during sister chromatid separation in mitosis by affecting the localization and activities of topoisomerase II (Figure 4-3F) (23,132,133). In addition, immunofluorescence microscopy studies in mammalian cells indicate that centromeres transiently co-localize with PML nuclear bodies in G2, a phenomena that is enhanced by proteasome inhibition (134). Although the functional significance of this association remains unexplored, telomere association with APBs is required for their maintenance in telomerase-deficient cells. It is therefore tempting to speculate by

analogy to APBs that interactions between centromeres and PML nuclear bodies in late G2 promotes SUMO-dependent reactions required for maintenance of centromeric DNA.

Future perspectives and conclusions

Sumoylation functions as a multifaceted regulator of chromatin structure, gene expression and genome integrity. Its utility resides in part in the ability of SUMO to elicit diverse downstream consequences following conjugation to different proteins. These consequences include affects on protein activity, localization, stability and interactions with a wide range of SIM-containing proteins. Understanding the rules that define the effects of sumoylation on specific chromatin-associated proteins, which are determined by the nature of the proteins themselves as well as any downstream interacting proteins, remains an important challenge for the field. In particular, understanding how sumoylation of different proteins mediates interactions with specific downstream SIM-containing proteins is critical. Specificity is likely to involve bivalent recognition of SUMO-modified proteins through downstream factors that contain both SIMs and motifs for recognizing the modified protein itself, as recently determined for Srs2 recognition of SUMO-modified PCNA (135). Specificity is also very likely to be determined and regulated by the intersection with other posttranslational modification pathways, including ubiquitylation, phosphorylation and acetylation. Understanding the crosstalk between sumoylation and other posttranslational modifications in greater detail is also an important challenge for the field.

Defining the role of sumoylation in controlling chromatin structure and function more specifically and at a molecular level will also require a more detailed

characterization of relevant SUMO-modified proteins. While the identification of SUMO-modified proteins with roles in chromatin structure and function has expanded greatly in the past several years, the functional effects of sumoylation on the majority of these proteins remain unknown. Characterizing the effects of sumoylation on individual proteins is often challenging, due in part to the relatively small fraction of most proteins modified at steady state. Other challenges involve identifying approaches for specifically affecting sumoylation of individual proteins or pathways. While many important studies linking sumoylation to chromatin structure and gene expression have relied on global activation or suppression of sumoylation, more targeted approaches are needed. The identification of new and functionally unique E3 ligases, as exemplified by Mms21, represents one avenue for developing more specific approaches. The identification of separation-of-function alleles of SUMO or SUMO pathway enzymes in yeast or other genetically tractable organisms could also prove valuable.

Finally, a more detailed understanding of the genome-wide interactions between SUMO (and SUMO pathway enzymes) and chromatin is needed. ChIP experiments on a small scale provided the surprising finding the SUMO is associated with the promoters of active but not repressed genes (35). Whole chromosome ChIP experiments, revealing genome-wide associations of SUMO during different stages of the cell cycle cell or under different cell growth conditions, could be particularly insightful and provide even more surprises.

In summary, we have reviewed the role of SUMO as a regulator of chromatin structure and function. In one important capacity, sumoylation regulates the assembly of multi-protein complexes on chromatin, including repressive complexes organized around

sites of DNA methylation and PcG bodies, as well as transcriptional regulatory complexes at gene promoters. A recurring theme from the reviewed studies is the dichotomous role of sumoylation. By facilitating the assembly of distinct complexes, sumoylation affects the chromatin environment in ways that can either activate or repress gene expression. In addition to facilitating protein complex assembly, sumoylation also affects proteins in multiple other ways by mediating changes in localization, stability or enzymatic activity. Thus, another recurring theme is the diverse and context-dependent effects of sumoylation on chromatin-associated proteins. Finally, we have reviewed the prominent role played by sumoylation in maintaining the integrity of repetitive heterochromatin domains, including telomeres, centromeres and rDNA loci. Collectively, the reviewed studies reveal the incredible versatility of sumoylation, which affects chromatin structure and function at multiple levels and through multiple mechanisms.

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Figure 4-1. The SUMO pathway and molecular consequences of sumoylation.

(A) SUMO is synthesized as a precursor, processed to a mature form by SUMO-specific isopeptidases and covalently conjugated to protein substrates via an E1, E2 and E3 enzyme cascade. Sumoylated protein substrates are demodified by SUMO-specific isopeptidases. **(B)** The molecular consequences of sumoylation (S) include protein targeting, alteration of protein or enzyme function, effects on protein stability, and effects on protein-protein interactions. Sumoylation can promote or antagonize protein stability by either blocking ubiquitylation of lysine residues or by promoting ubiquitylation (Ub) upon recognition by SUMO-targeted ubiquitin ligases (STUbL). Effects on protein-protein interactions may be modulated at multiple levels, including polymeric chain formation and intersection with other posttranslational modifications such as phosphorylation (P).

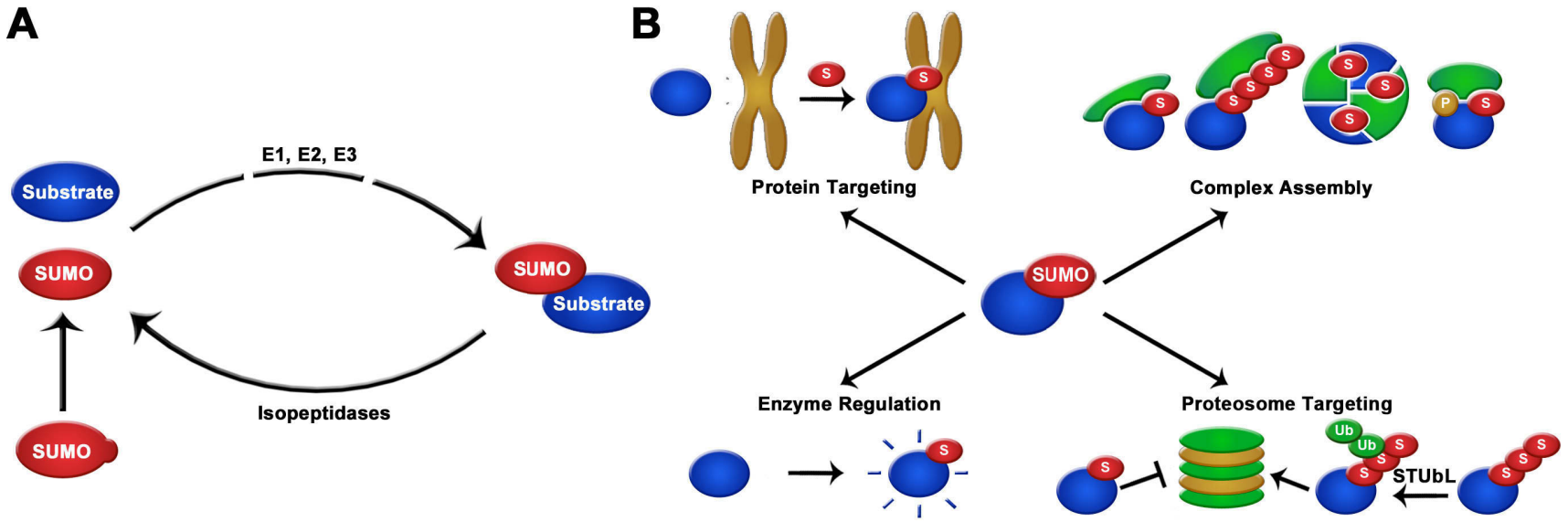

A
Substrate
SUMO
SUMO
E1, E2, E3
SUMO
Substrate
Isopeptidases
B
S
S
Protein Targeting
S
S
S
S
S
S
S
P
S
Complex Assembly
SUMO
Enzyme Regulation
S
Proteosome Targeting
S
Ub
Ub
S
S
S
STUbL
S
S
S

Figure 4-2. Sumoylation functions as an activator and a repressor of gene expression. (A) Sumoylation represses gene expression by promoting DNA methylation (yellow dots) through DNMT1 activation. **(B)** Sumoylation represses gene expression by facilitating assembly of repressive complexes on methylated DNA and at promoters. Sumoylation also inhibits the activities of transcription factors (TFs) and affects HDAC recruitment and function. **(C)** Sumoylation promotes the assembly of repressive PcG bodies. **(D)** Sumoylation promotes DNA demethylation and gene activation through mechanisms involving the SUMO-targeted ubiquitin E3 ligase activity of RNF4. **(E)** Sumoylation facilitates the assembly of complexes on chromatin that promote transcription. **(F)** Sumoylation positively influences RNA polymerase II (RNA Pol II) recruitment to constitutively active gene promoters.

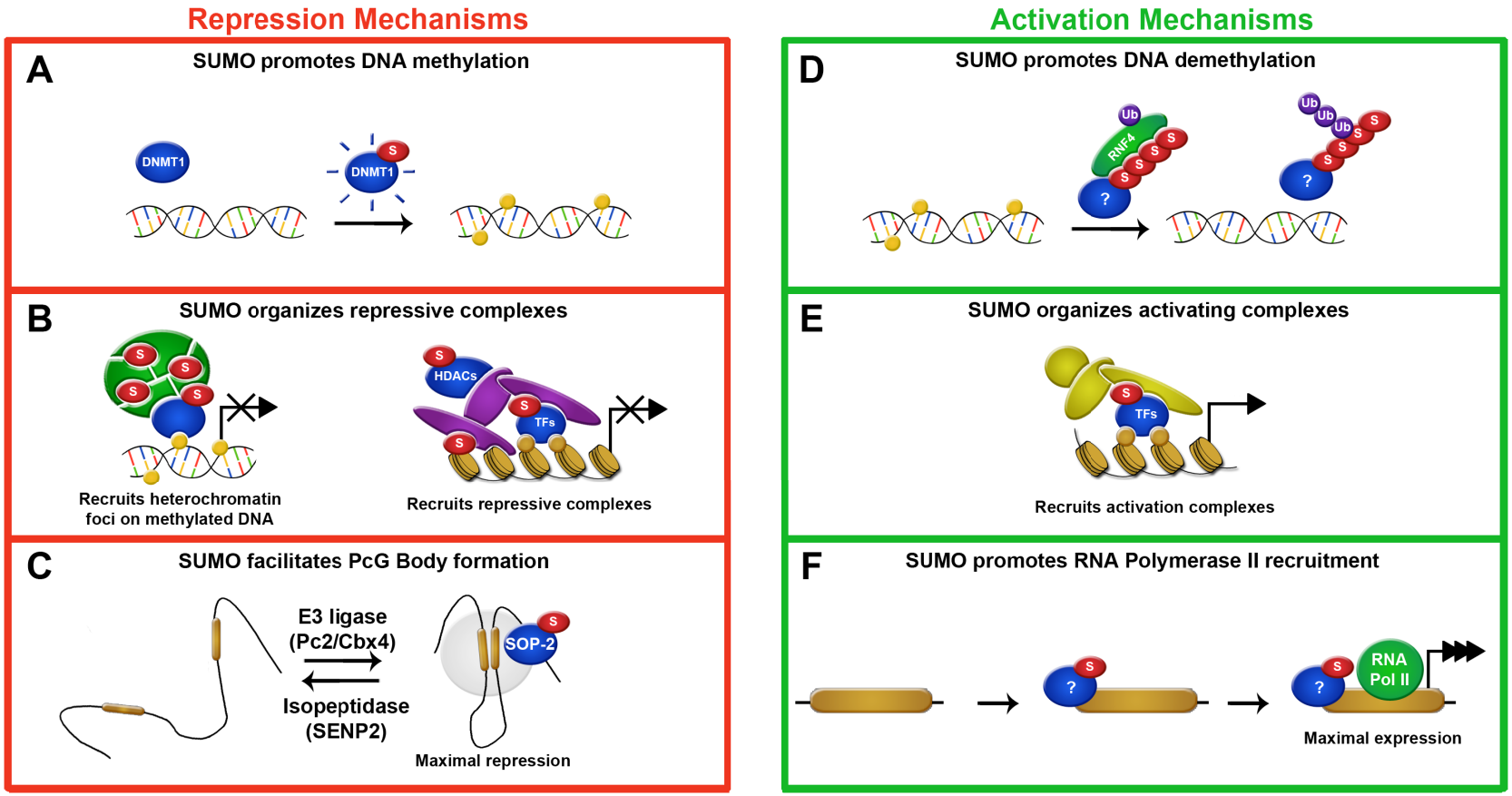
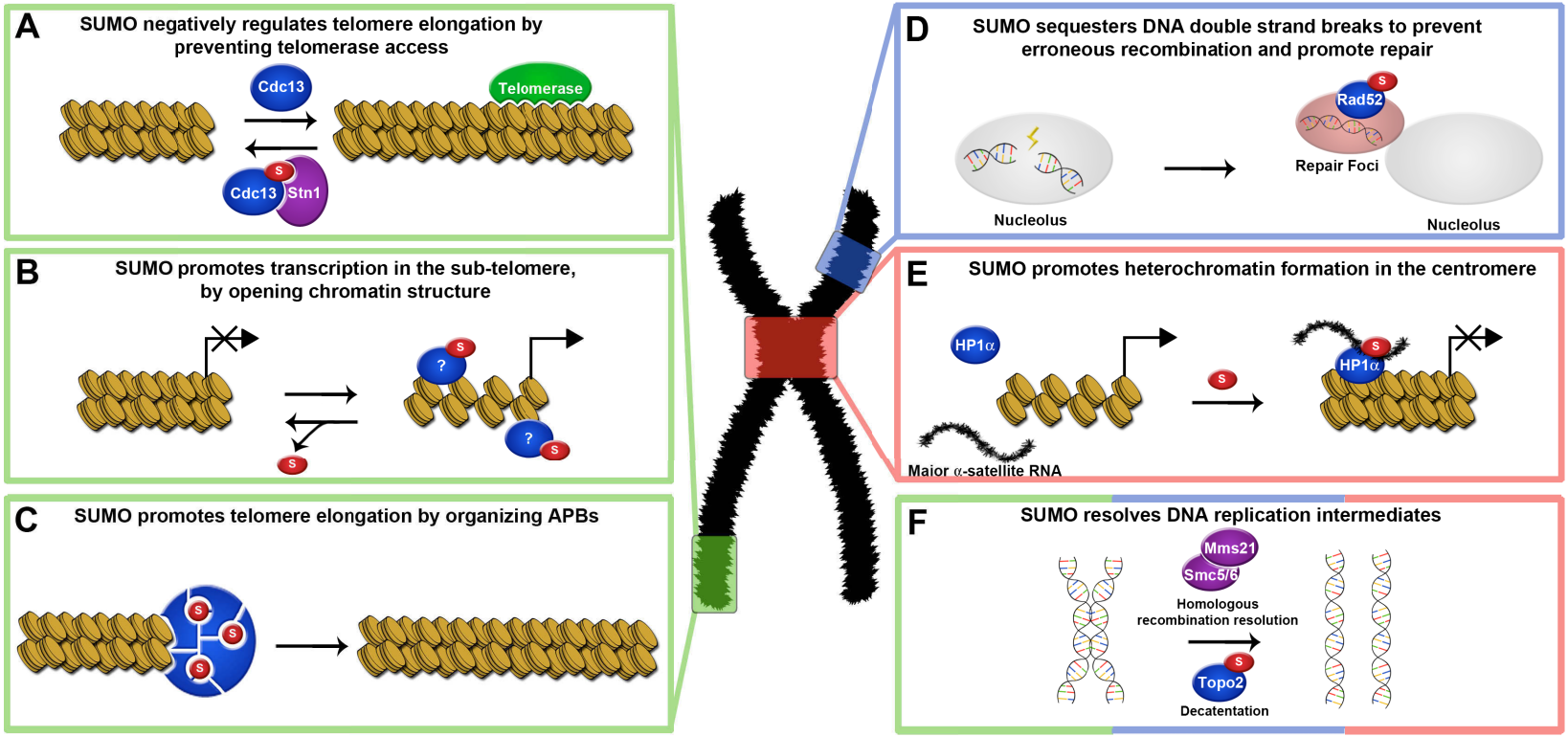

Repression Mechanisms
Activation Mechanisms
A
SUMO promotes DNA methylation
DNMT1
S
DNMT1
B
SUMO organizes repressive complexes
S
S
S
S
Recruits heterochromatin
foci on methylated DNA
S
HDACs
S
TFs
S
Recruits repressive complexes
C
SUMO facilitates PcG Body formation
E3 ligase
(Pc2/Cbx4)
Isopeptidase
(SENP2)
S
SOP-2
Maximal repression
D
SUMO promotes DNA demethylation
Ub
RNF4
S
S
S
S
?
Ub
Ub
Ub
S
S
S
S
?
E
SUMO organizes activating complexes
S
TFs
Recruits activation complexes
F
SUMO promotes RNA Polymerase II recruitment
S
?
S
?
RNA
Pol II
Maximal expression

Figure 4-3. Sumoylation maintains genome integrity at repetitive DNA domains. **(A)** Sumoylation limits telomere elongation by regulating interactions between Cdc13 and Stn1. **(B)** Sumoylation of unknown proteins affects sub-telomeric chromatin structure. **(C)** Sumoylation promotes the assembly of ALT PML nuclear bodies (APBs) essential for telomere maintenance in telomerase-deficient cells. **(D)** Sumoylation of Rad52 promotes the movement of DNA double-strand breaks from intranucleolar domains to the nucleolar periphery for optimal repair. **(E)** Sumoylation of HP1α regulates its association with α-satellite RNA and recruitment to centromeres. **(F)** Sumoylation functions to resolve DNA replication and repair intermediates within repetitive DNA domains and to promote decatenation.


A
SUMO negatively regulates telomere elongation by preventing telomerase access
Cdc13
Telomerase
S
Cdc13
Stn1
B
SUMO promotes transcription in the sub-telomere, by opening chromatin structure
S
?
S
?
S
C
SUMO promotes telomere elongation by organizing APBs
S
S
S
D
SUMO sequesters DNA double strand breaks to prevent erroneous recombination and promote repair
S
Rad52
Repair Foci
Nucleolus
Nucleolus
E
SUMO promotes heterochromatin formation in the centromere
HP1α
S
S
HP1α
Maior α-satellite RNA
F
SUMO resolves DNA replication intermediates
Mms21
Smc5/6
Homologous recombination resolution
S
Topo2
Decatentation

APPENDIX

AN ANALYSIS OF SENP1 INTERACTIONS AT THE NUCLEAR PORE COMPLEX AND FUNCTIONS IN MITOSIS

ABSTRACT

SENP1, a SUMO protease, is important for temporal and spatial control of sumoylation in mitosis. Notably, SENP1 localizes to nuclear pore complexes (NPCs) in interphase and centrosomes, mitotic spindles, and kinetochores in mitosis, where it is required for a timely metaphase to anaphase transition. In this study, we further characterized the targeting signals in SENP1 in interphase and mitosis. In addition, we utilized siRNA knockdown studies to characterize the molecular mechanism of SENP1 regulation of the metaphase to anaphase transition. We observed no defects in APC activation, sister chromatid cohesion, mitotic spindle structure, or general kinetochore protein recruitment after SENP1 knockdown. However, SENP1-depleted cells have an abnormal retention of Mad2, a spindle assembly checkpoint (SAC) protein, at kinetochores after bipolar attachment. Furthermore, the metaphase to anaphase transition delay observed after SENP1-depletion is rescued by a SAC inhibitor. Thus, in this study we have demonstrated that SENP1 is a novel regulator of the SAC.

INTRODUCTION

The SUMO protease SENP1 is often associated with cancer phenotypes, but the molecular mechanisms involved are still largely uncharacterized. SENP1 is overexpressed in prostate cancers and promotes cellular proliferation by affecting androgen receptor function (1-4). Furthermore, SENP1 overexpression has been observed in colon cancer and laryngeal squamous cell carcinomas (5,6). Notably, SENP1 is a driver of tumorigenesis in colon cancer cells because knockdown of SENP1 reduces tumor size (5). Thus, understanding the biochemistry and molecular mechanisms of SENP1 function is critical to learning how SENP1 drives tumorigenesis. In this appendix, I will address the functional role of SENP1 in mitosis, which may be one molecular mechanism by which SENP1 promotes cancer.

Two different groups have produced $SENP1^{-/-}$ mice that are embryonic lethal, demonstrating that SENP1 has essential, non-redundant functions with the other isopeptidases (7,8). $SENP1^{-/-}$ MEFs have a clear accumulation of SUMO-1 modified proteins and a more moderate increase in SUMO-2 modified proteins (7,8). Notably, the $SENP1^{-/-}$ embryonic lethality was partially rescued by crossing $SENP1^{+/-}$ mice into a $SUMO\text{-}1^{+/-}$ background, suggesting that the accumulated SUMO-1 modified proteins are the cause of some of the observed developmental defects (9). However, accumulations in SUMO-1 modified proteins are not the only defect in $SENP1^{-/-}$ animals, as these mice were stillborn or died shortly after birth.

Additional functions have been connected to SENP1 in various organisms and cell types. SENP1 knockout studies have shown that SENP1 functions in sister chromatid cohesion, placental development, the response to hypoxia, and mitochondrial biogenesis

(7,8,10,11). In addition, SENP1 siRNA knockdown studies have demonstrated that SENP1 is a cell cycle regulator, consistent with its role as a driver of tumorigenesis. SENP1-depletion results in cellular senescence or a G1 arrest in human foreskin fibroblasts and human colon cancer DLD-1 cells, respectively (5,12). Thus, SENP1 has a myriad of characterized functions, which likely reflects its cell-type specific isopeptidase activity and localization.

SENP1 binds SUMO-1, SUMO-2 and SUMO-3 and has the most robust endopeptidase activity of all the SENPs (13,14). Thus, SENP1 is expected to play an important role in the cleavage of precursor SUMOs to expose a GG motif at the C-terminus. SENP1 exhibits the strongest endopeptidase activity towards SUMO-1 and the weakest activity for SUMO-3 (13,15). Strikingly, chimeric proteins that swap the amino acids C-terminal to the GG on SUMO alter SENP1 specificity, demonstrating that divergence of the amino acids C-terminal to the GG are essential for SENP1 paralog-specific endopeptidase activity (13,15).

In addition to endopeptidase activity, SENP1 also has robust isopeptidase activity important for the cleavage of SUMO from protein substrates. Interactions between SENP1 and SUMO occur within the relatively conserved C-terminus of mature SUMO and the active site cleft of SENP1, allowing SENP1 to bind and deconjugate all three SUMO paralogs (16). However, SENP1 has a preference for deconjugating the different SUMO paralogs from substrates i*n vitro*, with the highest activity against SUMO-2 modified proteins and the lowest activity against SUMO-1 (13). In almost all cell types, SENP1 is the most robust isopeptidase for SUMO-1 modified proteins and also has very strong activity for SUMO-2 modified proteins (17).

Finally, there have been a few other interesting observations about SENP1 catalytic activity. First, overexpression of the catalytic mutants of SENP1 C603A or C603S results in an accumulation of SUMO-1 modified proteins (18,19). These findings suggest that the catalytically dead SENP1 proteins are binding substrates and preventing their deconjugation by other isopeptidases. This hypothesis is supported by the observation that SENP1 C603S can be co-purified with large numbers of SUMO-1 modified proteins (18).

Precisely what regulates the isopeptidase and endopeptidase activities of SENP1 are still unknown. Three different studies have demonstrated that SENP1 is modified by SUMO-1 and SUMO-2/3 (9,18,20). SENP1 sumoylation is most clearly observed with catalytically dead versions of SENP1, suggesting auto-desumoylation occurs (20,21). However, the site of SENP1 sumoylation and its functional significance has yet to be characterized. Interestingly, SENP1 activity may also be regulated by protein stability. For example, Nup153 depletion causes a reduction in SENP1 protein levels (20). Because SENP1 and Nup153 interact, this result suggests that SENP1-Nup153 protein-protein interactions are required for SENP1 stability (20). Further studies are required to fully understand how SENP1 is regulated through other posttranslational or protein stability mechanisms.

In addition to its catalytic domain, SENP1 contains an N-terminal domain that is critical for its substrate specificity and localization to nuclear pore complexes (NPCs) in interphase (Chapter 3). However, the targeting signals and protein-protein interaction domains within the SENP1 N-terminus are still largely uncharacterized. Amino acids 171-177 encode a nuclear localization signal (NLS) that is necessary but not sufficient for

nuclear import, while amino acids 634-644 encode a nuclear export signal (NES) (18,19). Thus, SENP1 likely shuttles between the cytoplasm and nucleus, which is supported by findings showing SENP1 localization varies from nucleoplasmic to cytoplasmic in different cell types (19,21,22). In addition, SENP1 interacts with the nucleoporins Nup153, the Nup107-160 complex, and Nup358 (Chapter 3 and (20)), suggesting that SENP1 targeting to NPCs is likely mediated by a combination of nucleoplasmic shuttling and interactions with nucleoporins. However, a more careful analysis of the SENP1 N-terminus is required to determine which interaction domains are critical for targeting.

We previously demonstrated that SENP1 localizes to NPCs and that SENP1 knockdown results in a delay in the metaphase to anaphase transition (Chapter 3). In this study, we sought to further characterize the interactions of SENP1 with the NPC because multiple nucleoporins, including the Nup107-160 complex, Nup358, Tpr, Nup153, and Nup98, have critical mitotic functions at kinetochores and the mitotic spindle (23). We demonstrated that SENP1 localizes to the nucleoplasmic face of the NPC and has two independent NPC-targeting elements. In addition, we evaluated the function of SENP1 in four potential molecular processes that could cause a metaphase to anaphase transition delay: sister chromatid cohesion, anaphase promoting complex activation, mitotic spindle and kinetochore assembly, and the stability of the spindle assembly checkpoint (SAC). We demonstrated that the SENP1 knockdown phenotype is dependent on the SAC and causes abnormal retention of Mad2, a SAC protein, at kinetochores after proper chromosome alignment. Thus, our findings demonstrate that SENP1 is a novel regulator of SAC inactivation and function.

MATERIALS AND METHODS

Antibodies

GFP rabbit polyclonal antibodies were produced as described previously (24). The SENP1 antibody, a gift from Dr. Mary Dasso (National Institute of Health, Bethesda, MD), was generated by injecting rabbits with GST-SENP1 (273-449) as previously described (20). Antibodies were affinity purified using appropriate antigens and standard protocols.

Remaining antibodies were obtained from the following sources: Tpr (5E10), Nup358 (14E6), Nup358 C-288, Hec1 (BD Transduction – 611040); CENP-E (Active motif - 39620); CENP-F (BD 610768); INCENP (Active motif – 39259); CREST human auto-antibodies were a generous gift from Dr. Ted Salmon (University of North Carolina, NC); Mad2 was a generous gift from Dr. Ted Salmon (University of North Carolina, NC); Cyclin B1 (Santa Cruz sc-245), SUMO-2/3 (8A2), Tubulin (DM1A) Sigma T9026; TRIM28 (20A1) Enzo ADI-KAM-TF200-D; mAb 414 – BAbCO.

Plasmid constructs

SENP1 GFP-tagged and mCherry-tagged expression constructs were obtained as previously described (Chapter 3). The GFP-SENP1 ΔNES (1-633) construct was generated using site directed mutagenesis to create a stop codon at amino acid 634. The GFP-SENP1 fragments (1-40, 41-153, 154-272, 273-449, 320-400 and 401-644) were a gracious gift from Mary Dasso (National Institute of Health, Bethesda, MD).

Cells, cell culture, transfection, RNA interference

HeLa cells stably expressing YFP-Histone H2B were a gift from Dr. Andrew Holland (Johns Hopkins University School of Medicine, Baltimore, MD). HeLa and HEK 293T cells were maintained at 37°C in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 10 mM HEPES (pH 8.0) and 1% penicillin-streptomycin. Cells were transfected with the indicated plasmids at a confluency of 40-50% using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For RNA interference, cells were grown to 40-50% confluency and then transfected using RNAiMax (Invitrogen, Carlsbad, CA). siRNA oligos were used at a final concentration of 20 nM. siRNA oligos included: scramble control (5'-CUUCCUCUCUUUCUCUCCCUUGUGA-3'); SENP1 oligo(a) (5'-UCCUUUACACCUGUCUCGAUGUCUU-3') and SENP1 oligo(b) (5'-GCAAAUGGCCAAUGG AGAAAUUCUA-3'). For nocodazole synchronizations, cells were treated with 0.1 μg/ml nocodazole overnight prior to harvest. For some experiments, nocodazole was washed out with fresh media for two hours prior to harvest. Cells were harvested for immunoblotting, immunofluorescence microscopy, or FACs 48 hours after transfection. For reversine experiments, cells were treated with 1 μM reversine for 1.5 hours prior to harvest.

Immunoblotting

Immunoblot analysis was performed using enzyme-linked chemiluminescence ECL-Prime reagent (GE Healthcare, Silver Spring, MD).

FACS Analysis

Cells were trypsinized 48 hours after knockdown and processed for FACs analysis as described previously (25). Briefly, cells were spun for 5 minutes at 200 x g and resuspended in 500 µl PBS with a glass pipette. Cells were vortexed 3 times in 5 second pulses and added dropwise to 4.5 ml 70% ethanol on ice and rocked at 4°C for at least 2 hours for fixation. Cells were spun for 5 minutes at 200 x g, resuspended in PBS, and centrifuged again. Cells were resuspended in freshly prepared staining solution (0.1% (v/v) Triton X-100, 0.2 mg/ml RNAse A, 20 µg/ml Propidium Iodide (Invitrogen)) for 30 minutes at 37°C prior to FACs analysis. Samples were processed on a FACS Calibur flow cytometer (BD Biosciences) using a 488 nm laser according to manufacturer protocols. Data was collected for 10,000 cells and analyzed using the Cell Quest software.

Geimsa Staining of Chromosomes

HeLa cells were transfected and synchronized. 12 hours after transfection, cells were treated with 2.5 mM thymidine for 24 hours, followed by 12 hour wash out and then harvested by mitotic shake off and trypsinization approximately 48 hours after the initial transfection. Cells were spun at 200 x g for 10 minutes at 4°C and all media except 500 µl was aspirated. The pellet was flicked to gently resuspend the cells and 7 ml of 37°C 0.8% sodium citrate was added to cells dropwise while tapping the tube. Cells were incubated at room temperature for 10 minutes and centrifuged at 200 x g for 10 minutes at 4°C. The supernatant was aspirated except 500 µl and the pellet was flicked to resuspend cells. 7 ml of freshly prepared Carnoy's fixative was added (75% methanol,

25% acetic acid) dropwise. Cells were incubated at room temperature for 10 minutes and centrifuged at 200 x g for 10 minutes at 4°C. Resupension and incubation in Carnoy's fixative was repeated 2 more times. Cells were resuspended in a final volume of 500 µl Carnoy's fixative and dropped onto slides. Slides were placed on a wet paper towel in a 37°C water bath and cells were dropped from the standing height of a person on a stool. Slides were dried in a fume hood for approximately 10 minutes and stained in 1:20 diluted Geimsa stain (Sigma – GS500) for 30 minutes. Slides were rinsed twice in water, air-dried, and a mounting medium was added prior to sealing the slide with a cover slip.

Immunofluorescence microscopy

HeLa or HEK 293T cells were cultured on glass coverslips. Unless otherwise stated, cells were fixed in 2% formaldehyde for 30 min and permeabilized in 0.2% Triton-X 100 for 7 min at RT. For digitonin permeabilization in interphase, cells were fixed in 2% formaldehyde for 30 min and permeabilized in 10 µg/ml digitonin in PBS for 10 minutes. Localization of mitotic GFP-SENP1 was examined by pre-extracting cells in 20 µg/ml digitonin in buffer containing (200 mM HEPES (pH 6.5), 110 mM potassium acetate, 20 mM magnesium acetate, 1 µg/ml leupeptin and pepstatin A, 20 µg/ml aprotinin, and 1 mM PMSF) for 15 min at RT and then fixing in 2% formaldehyde in PBS for 30 min. For localization of kinetochore proteins, cells were fixed in 3.5% paraformaldehyde in PBS for 7 min and permeabilized in 0.5% Triton-X 100 in PBS for 20 min at RT. Immunostaining was carried out as previously described using secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (Life Technologies, Grand Island, NY), unless otherwise noted (26). Images were acquired

using the Zeiss Observer Z1 fluorescence microscope with a Zeiss Plan-Apochromat 63X objective (numerical aperature = 1.40) and an Apotome VH optical sectioning grid. Images were obtained using a Zeiss Axiocam MRm camera and processed using AxioVision Software Release 4.8.2 (Zeiss, Gena, Germany).

RESULTS

SENP1 localizes to the nuclear side of the NPC and contains two independent NPC targeting elements

Although we have localized SENP1 to NPCs in interphase and to mitotic spindles, centrosomes and kinetochores in mitosis, the precise associations of SENP1 remain uncharacterized. First, we determined if SENP1 is associated with the cytoplasmic or nuclear face of the NPC. Cells were fixed and permeabilized with Triton-X 100, which exposes epitopes in both the nucleus and cytoplasm, or with digitonin, which only exposes cytoplasmic epitopes. HeLa cells were stained for endogenous SENP1 and Tpr, a protein on the nucleoplasmic face on the NPC, or Nup358, a component of the cytoplasmic filaments of the NPC (Figure 5-1A). Endogenous SENP1 was only detectable after Triton-X 100 permeabilization, indicating that SENP1 localizes specifically to the nucleoplasmic face of the NPC (Figure 5-1A).

Because many of our experiments will rely on GFP-SENP1 constructs, we investigated the localization of overexpressed wild-type GFP-SENP1 at NPCs. For these studies, we transfected HeLa cells with GFP-SENP1 and identified SENP1-expressing cells by the fluorescent signal of GFP. Furthermore, we utilized indirect immunofluorescence using a GFP-specific antibody with either a Triton-X 100 or digitonin permeabilization to determine the precise localization of GFP-SENP1. Overexpressed GFP-SENP1 localized to the nuclear face of the NPC, consistent with the observations for endogenous SENP1 (Figure 5-1B).

The targeting signals affecting SENP1 localization are still largely uncharacterized. Therefore, we utilized a series of SENP1 fragments fused to GFP to

identify regions important for SENP1 NPC targeting (Figure 5-2A). HeLa cells were transfected with constructs coding for the GFP-SENP1 fusions and stained with mAb 414 to detect NPCs. Two SENP1 fragments, including amino acids 154-272 and 273-449 localized to NPCs, indicating that each contains an independent targeting element (Figure 5-2B). The signal within residues 273-449 of SENP1 was further narrowed to amino acids 321-400 (Figure 5-2B), which has a comparable signal to the larger fragment, suggesting there is a single targeting signal within the amino acid 273-449 sequence. In conclusion, we demonstrated that SENP1 localizes to the nucleoplasmic face of the NPC and that this localization is mediated by two independent elements in the N-terminus.

Mitotic targeting of SENP1 is mediated by three independent elements

During mitosis, SENP1 redistributes from NPCs to centrosomes, the mitotic spindle and kinetochores (Chapter 3). Thus, we next sought to investigate the signals affecting SENP1 localization to these mitotic structures. We have hypothesized that GFP-SENP1 targeting to mitotic structures is dependent on interactions with nucleoporins (Chapter 3). We therefore investigated whether regions of SENP1 critical for NPC-targeting were also necessary and sufficient for mitotic targeting (Figure 5-3).

HeLa cells were transfected with GFP-SENP1 or GFP-SENP1 fragments and stained with antibodies specific for α-tubulin to highlight the mitotic spindle and CREST to identify the centromere. We identified three SENP1 fragments, containing amino acids 41-153, 273-449, and 401-644, that were sufficient for centrosome, spindle and kinetochore localization. Surprisingly, only the SENP1 fragment containing amino acids 273-449 was also sufficient for NPC targeting (Figure 5-2 and 5-3). Thus, we

demonstrated that GFP-SENP1 localization to kinetochore contains three independent targeting elements for its mitotic localization.

SENP1 knockdown does not affect APC activation or cohesion stability

Next, we sought to further characterize the potential causes of the metaphase to anaphase transition delay observed with SENP1 knockdown (Chapter 3). One potential mechanism may involve improper activation of the anaphase promoting complex (APC) in SENP1-depleted cells, resulting in hyper-stable cohesion. However, a recent analysis of $SENP1^{-/-}$ DT40 chicken cells showed that the absence of SENP1 destabilized cohesion. Thus, we characterized the effects of SENP1-depletion on APC activation and cohesion stability.

To evaluate APC activity, we analyzed cyclin B1 protein levels, a known APC target, at various stages of mitosis. As predicted, cyclin B1 staining was very high in the early mitotic stages, declined at the metaphase to anaphase transition, and was completely absent from anaphase cells (Figure 5-4A) (27). Control and SENP1-depleted cells exhibited comparable cyclin B1 staining, indicating that APC activation is normal in the absence of SENP1 (Figure 5-4A).

Next, we investigated the effects of SENP1-depletion on sister chromatid cohesion maintenance. We evaluated the ability of SENP1-depleted cells to maintain cohesion when cultured in the presence of nocodazole. Control and SENP1-depleted cells were both synchronized to similar levels following overnight nocodazole treatments, indicating the absence of SENP1 does not promote mitotic slippage (Figure 5-4B). In addition, we characterized cohesion stability after SENP1-depletion in the absence of

microtubule destabilizing agents by conducting a chromosome spread analysis. Under normal mitotic cycling, chromosomes can be observed with sister chromatid cohesion intact, partially separated or completely separated (Figure 5-4C). Over 200 cells were quantified for control and SENP1-depleted cells, but no significant differences in cohesion distribution were observed (Figure 5-4D). In summary, our studies exhibited no defects in APC activation or cohesion stability after SENP-1 depletion.

SENP1 knockdown does not cause morphological changes in the mitotic spindle or kinetochore structure

SENP1 localizes to the mitotic spindle, suggesting a possible function for desumoylation in spindle structure or function. Thus, we analyzed the mitotic spindle morphology in SENP1-depleted cells.

First, we investigated the general spindle morphology by α-tubulin staining. However, SENP1-depleted cells had mitotic spindle structures comparable to control cells (Figure 5-5A). Next, we sought to investigate if mitotic spindles in SENP1-depleted were unusually sensitive to treatment with a microtubule destabilizing agent. We treated control and SENP1-depleted cells with nocodazole overnight followed by a 2 hour release to allow mitotic spindle reformation. Control and SENP1-depleted cells exhibited mitotic spindles in various stages of reformation, indicating SENP1-depeletion did not cause enhanced nocodazole-sensitivity (Figure 5-5B). Finally, we investigated whether SENP1 is required for the proper spindle positioning within mitotic cells. Previous studies have shown β1-integrin-depletion results in the metaphase plate formation very close to the cell cortex (28). Thus, we utilized TRIM28-staining to visualize the

cytoplasm and determine the position of the metaphase plate relative to the cell cortex (Figure 5-5C). No changes in metaphase plate position were observed SENP1-depleted cells compared to controls. In conclusion, our analysis did not detect any obvious defects in mitotic spindle morphology.

Even though mitotic spindle morphology was normal in SENP1-depleted cells, defective kinetochore-microtubule attachments could also cause a metaphase to anaphase transition delay. Thus, we analyzed the localization of eight different centromere and kinetochore proteins in prometaphase, metaphase and anaphase to characterize kinetochore assembly and structure. The recruitment and dynamics of CENP-E, CENP-F, CREST (CENP-A, B and C), Hec1, INCENP, and Nup358 were unaffected by SENP1-depletion (Figure 5-6). Thus, we detected no obvious defects in the general centromere and kinetochore structure.

The metaphase to anaphase transition delay observed in SENP1-depleted cells is caused by improper inactivation of the SAC

Previous studies have shown that improper inactivation of the SAC results in a metaphase to anaphase transition delay comparable to our observations after SENP1-depletion (29-31). Therefore, we utilized a recently identified inhibitor of the SAC, reversine, to determine if the mitotic delay observed with SENP1-depletion depends on SAC activity (32). First, we demonstrated that reversine treatment inhibits SAC activity by causing mitotic slippage in the presence of nocodazole (Figure 5-7A). HeLa cells were treated with two independent oligos specific for SENP1 for 48 hours and treated with reversine to inactivate the SAC for 1.5 hours prior to harvest. Strikingly, the 2-3 fold

increase in mitotic index observed in SENP1-depleted cells was rescued by reversine treatment, indicating that the SAC activity is required for the SENP1 knockdown mitotic delay phenotype (Figure 5-7B).

Next, we evaluated the recruitment and release of Mad2, a core SAC component, in SENP1-depleted cells. In the early phases of mitosis, Mad2 is recruited to kinetochores unattached to the mitotic spindle, which was observed in control and SENP1-depleted cells (Figure 5-7C). Following proper chromosome alignment, Mad2 is released from kinetochores, which is clearly observed in the control cells (Figure 5-7C). Notably, Mad2 foci are still detectable at kinetochores of chromosomes that have aligned on the metaphase plate in SENP1-depleted cells, indicating that Mad2 release is delayed. However, there is not a complete block in Mad2 release because Mad2 is no longer detectable on kinetochores in anaphase of SENP1-depleted cells (Figure 5-7C). Thus, we have demonstrated that the metaphase to anaphase transition delay observed after SENP1 depletion requires the SAC, and that Mad2 is improperly retained at kinetochores.

SENP1 knockdown results in a delay in S phase progression

We have characterized the function of SENP1 in mitotic progression, but previous studies have demonstrated that SENP1 knockdown caused cells to arrest in G1 or senesce (5,12). Thus, we investigated if there were any other cell cycle delays in our SENP1-depleted HeLa cells. We utilized FACs analysis to evaluate the DNA content of cells after SENP1 knockdown. There was a statistically significant increase in the number of cells in S phase, indicating that there is a prolonged S phase after SENP1 depletion (Figure 5-8). A reciprocal reduction in the number of cells in G1 was also observed.

Surprisingly, a statistically significant increase was not detected with both SENP1-specific oligos in the G2/M population. However, the mitotic progression phenotype after SENP1 depletion we characterized by live cell imaging was only a 1 to 2 hour delay and thus, was likely below the threshold of detection by this assay. Further analysis is required to determine the functional significance of the prolonged S phase observed in the absence of SENP1.

SENP1 has unique localizations in different cell types because of its dynamic nuclear shuttling

In this study, we focused on the function of SENP1 in HeLa cells, but SENP1 has been reported to localize to nuclear speckles, the nucleoplasm, the cytoplasm, or the NPC depending on the cell type analyzed (18,19,22) (Chapter 3). Thus, we sought to investigate this cell type dependent localization of SENP1 further. Kim and colleagues previously demonstrated that amino acids 634-644 constitute a NES that is necessary and sufficient for SENP1 nuclear export. Furthermore, the NES was responsible for GFP-SENP1 localization to the cytoplasm in CV-1 cells, as GFP-SENP1 ΔNES, a construct encoding amino acids 1-633 of SENP1, exhibited a nucleoplasmic localization (19).

Thus, we determined the cellular localization of GFP-SENP1 in HeLa and HEK 293T cells. In both cell types, SENP1 was enriched at the nuclear rim, indicative of NPC staining (Figure 5-9A). However, GFP-SENP1 also exhibited a strong cytoplasmic localization in HEK 293T cells under all expression levels, while HeLa cells expressing low to moderate levels of GFP-SENP1 exhibited a more restricted NPC localization (Figure 5-9A).

Next, we sought to determine if SENP1 nuclear shuttling was important for the differential localization observed in HeLa and HEK 293T cells. Thus, we transfected both cell types with GFP-SENP1 WT or GFP-SENP1 ΔNES and conducted a fluorescence microscopy analysis. Strikingly, the cytoplasmic localization in HEK293T cells was significantly diminished, with the majority of GFP-SENP1 ΔNES localizing to the nuclear rim (Figure 5-3B). SENP1 localization in HeLa cells, on the other hand, was unaffected (Figure 5-3B). Thus, the cytoplasmic localization of GFP-SENP1 in HEK 293T cells is dependent on NES-mediated nuclear export of SENP1.

DISCUSSION

SENP1 localizes to NPCs in interphase and relocalizes to kinetochores, centrosomes, and the mitotic spindle during mitosis. Furthermore, SENP1-depletion results in a metaphase to anaphase transition delay that is rescued by WT but not the catalytically dead versions of SENP1 (Chapter 3). In this study, we further characterized the localization of SENP1 in interphase and mitosis and determined that the mechanism for SENP1-dependent regulation of the metaphase to anaphase transition involves the SAC.

Our studies demonstrated that SENP1 localizes to the nuclear face of NPCs (Figure 5-1). SENP1 is known to bind Nup153, Nup358 and the Nup107-160 subcomplex, but only Nup153 and Nup107-160 are found on the nucleoplasmic side of the NPC (20,33,34) (Chapter 3). Thus, we would speculate that Nup153 and Nup107-160 are important for SENP1 NPC localization. Intriguingly, two core SAC proteins Mad1 and Mad2 also localize to the nuclear face of NPCs in interphase and in this study, we demonstrated that SENP1 is an important regulator of the SAC (Figure 5-7) and (35). These findings suggest that SENP1 may regulate the localization or function of Nups or Mad proteins on the nucleoplasmic face of NPCs in interphase.

In addition, we demonstrated that SENP1 has two independent NPC targeting signals: amino acids 154-272 and 321-400. Intriguingly, amino acids 186-199 (VQEEEREIYRQLLQ) of the SENP1 N-terminus are highly conserved with the region of SENP2 known to bind the Nup107-160 subcomplex (24). Thus, we hypothesize that the region of SENP1 between amino acids 154-272 promotes NPC-targeting through interactions with the Nup107-160 subcomplex. The region of SENP1 between amino

acids 321-400 may mediate interactions with Nup153 or the Mad proteins. In the future, we can utilize the two NPC targeting elements of SENP1 in co-immunoprecipitation experiments to determine the protein-protein interactions that mediate SENP1 association with NPCs.

We mapped three targeting elements of SENP1 sufficient for mitotic localization to spindles, centrosomes, and kinetochores: amino acids 41-152, 273-449, and 401-644. Surprisingly, the putative Nup107-160 subcomplex binding region, including amino acids 154-272, was not sufficient for mitotic targeting of SENP1 even though the Nup107-160 subcomplex localizes to the same mitotic structures as SENP1 (36). However, it will be critical to conduct co-immunoprecipitation experiments using the SENP1 fragments from mitotic cell extracts to determine which Nups or potentially SAC proteins are binding SENP1 and potentially promoting recruitment to kinetochores, spindles and centrosomes.

We previously demonstrated that a chimeric protein that contained the SENP2 N-terminus and the SENP1 catalytic domain (amino acids 419-644) called $SENP2_N1_{CAT}$ does not localize to centrosomes or mitotic spindles (Chapter 3). Thus, it was surprising that amino acids 401-644 were sufficient for SENP1 kinetochore, centrosome and spindle targeting in mitosis (Chapter 3). There are multiple interpretations for this result. First, this finding could suggest that the amino acids 401-418, which were not in the chimeric protein $SENP2_N1_{CAT}$, contain a critical targeting element for centrosomes and spindles. This is supported by the observation that the smaller SENP1 fragment containing amino acids 320-400 was insufficient for centrosome and spindle targeting. However, we cannot rule out that the SENP2 N-terminus could have a dominant targeting element that masked the spindle and centrosome targeting elements in the SENP1 catalytic domain. In

summary, these findings warrant further investigation of the SENP1 mitotic spindle and centrosome targeting element potentially present between amino acids 400-418 of the N-terminus or within the catalytic domain of SENP1.

Next, we sought to characterize the molecular mechanism involved in the metaphase to anaphase delay observed in SENP1 knockdown cells. No defects were observed with APC activation, sister chromatid cohesion, mitotic spindle morphology, or general kinetochore structure in the absence of SENP1. Notably, we demonstrated that the delayed mitotic progression observed after SENP1-depletion depends on the SAC. Furthermore, the absence of SENP1 causes retention of Mad2 at kinetochores after bipolar attachment, which indicates that the SAC inactivation is defective. Intriguingly, the SAC proteins Mad1 and Mad2 are known to bind the nucleoplasmic side of the NPC, which mirrors SENP1 interphase localization (35). Thus, we hypothesize that SENP1 interacts with SAC proteins throughout the cell cycle and regulates their function at the metaphase to anaphase transition.

There are three molecular mechanisms described in the literature for a metaphase to anaphase transition delay caused by abnormal retention of SAC proteins at kinetochores. Mps1 is a kinase critical for SAC protein recruitment to kinetochores. Intriguingly, when Mps1 is artificially tethered to kinetochores, a SENP1-like phenotype is observed (31). Of note, Mps1 was identified as a SENP1-interacting protein by preliminary BioID and mass spectrometry analysis, further suggesting that SENP1 may regulate Mps1 activity (Unpublished, Dr. Brian Raught, Univeristy of Toronto). In addition, a SENP1-like phenotype occurs after knockdown of dynein. Dynein facilitates the removal of Mad2 from properly bioriented kinetochores and dynein knockdown

results phenotype that mimics SENP1-depletion (30). Finally, knockdown of Spindly, a relatively uncharacterized SAC protein, blocks proper dynein recruitment and thus phenocopies a dynein knockdown (29). In the future, we will investigate if SENP1 interacts with Mps1, dynein or spindly, which could provide insights into precisely how SENP1 regulates SAC function.

In summary, our investigations have identified SENP1 as a novel regulator of the SAC. The common localization of SENP1 and Mad1 and Mad2 to the nucleoplasmic face of the NPC in interphase and the abnormal retention of Mad2 at bi-oriented kinetochores after SENP1-depletion suggests that SENP1 interacts with SAC proteins throughout the cell cycle. Further investigation of the SUMO modification of SAC proteins throughout the cell cycle and after SENP1-depletion will be vital in further characterizing the molecular mechanism of SENP1 SAC regulation.

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Figure 5-1. SENP1 localizes to the nucleoplasmic face of the NPC. (A) HeLa cells were fixed and permeabilized with either Triton-X 100 (TNX-100) or digitonin. Cells were stained using antibodies specific for SENP1, Tpr or Nup358 and analyzed by immunofluorescence microscopy. **(B)** HeLa cells were transfected with GFP-SENP1, fixed and permeabilized with either TNX-100 or digitonin 48 hours after transfection. Cells were stained using antibodies specific for GFP and analyzed by immunofluorescence microscopy.

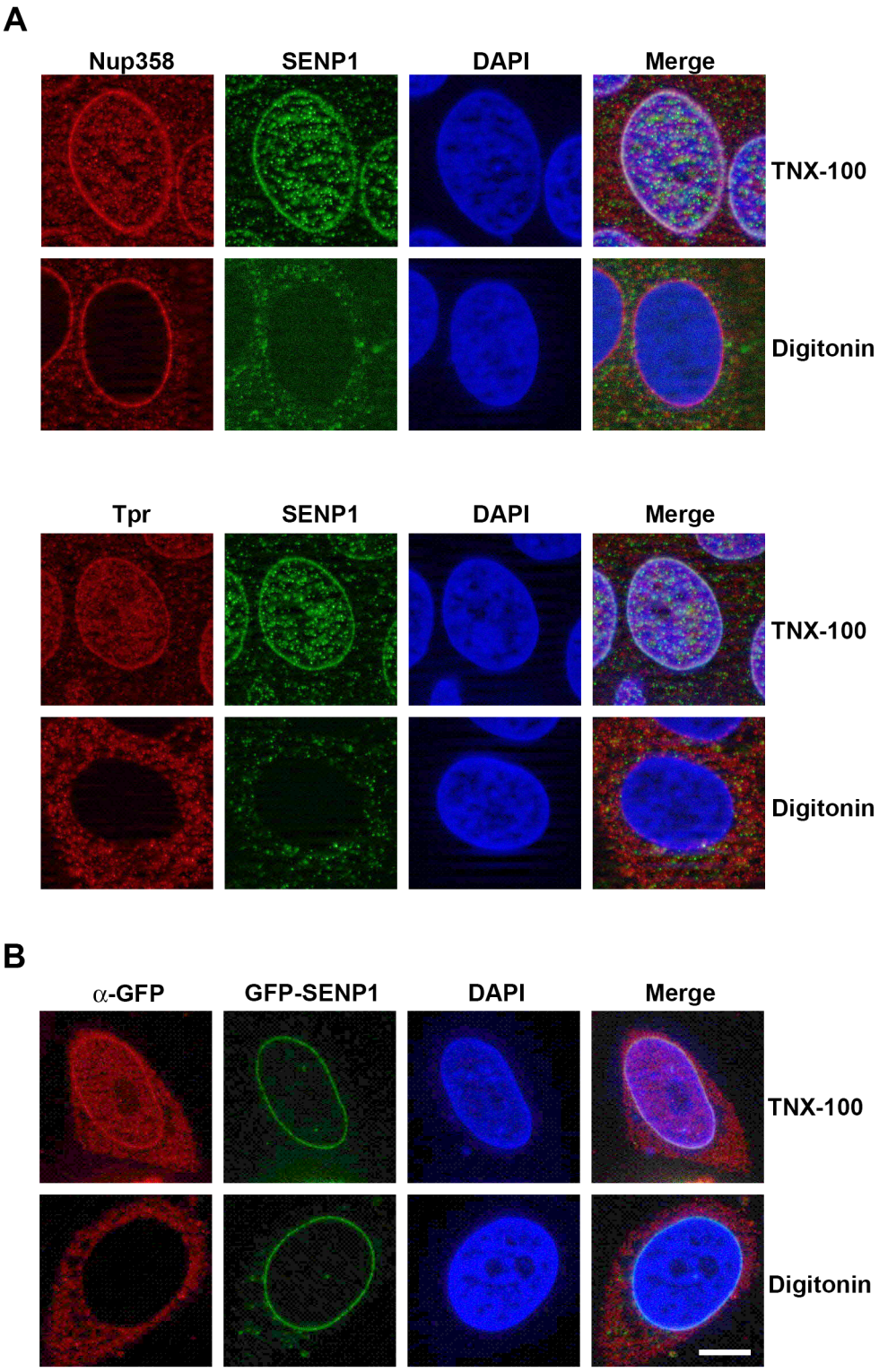

A
Nup358
SENP1
DAPI
Merge
TNX-100
Digitonin
Tpr
SENP1
DAPI
Merge
TNX-100
Digitonin
B
α-GFP
GFP-SENP1
DAPI
Merge
TNX-100
Digitonin

Figure 5-2. SENP1 targeting to the NPC is mediated by two independent targeting elements. (A) Cartoon schematic showing the known domain structure and targeting elements of SENP1. The SENP1 fragments utilized in this study are also depicted. The N-terminal domain is shown in blue and the catalytic domain (CAT) is shown in green. The following elements are also highlighted: the nuclear localization signal (NLS) is yellow, the catalytic cysteine is cyan (C603) and the nuclear export signal (NES) is red. **(B)** HeLa cells were transfected with WT GFP-SENP1 or GFP-SENP1 fragments. Cells were harvested 48 hours after transfection, fixed, permeabilized with TNX-100 and stained with mAb 414, which recognizes Nup62, Nup153, Nup214 and Nup358. DNA was stained with DAPI. Cells were visualized by immunofluorescence microscopy. Bar = 10 μm.

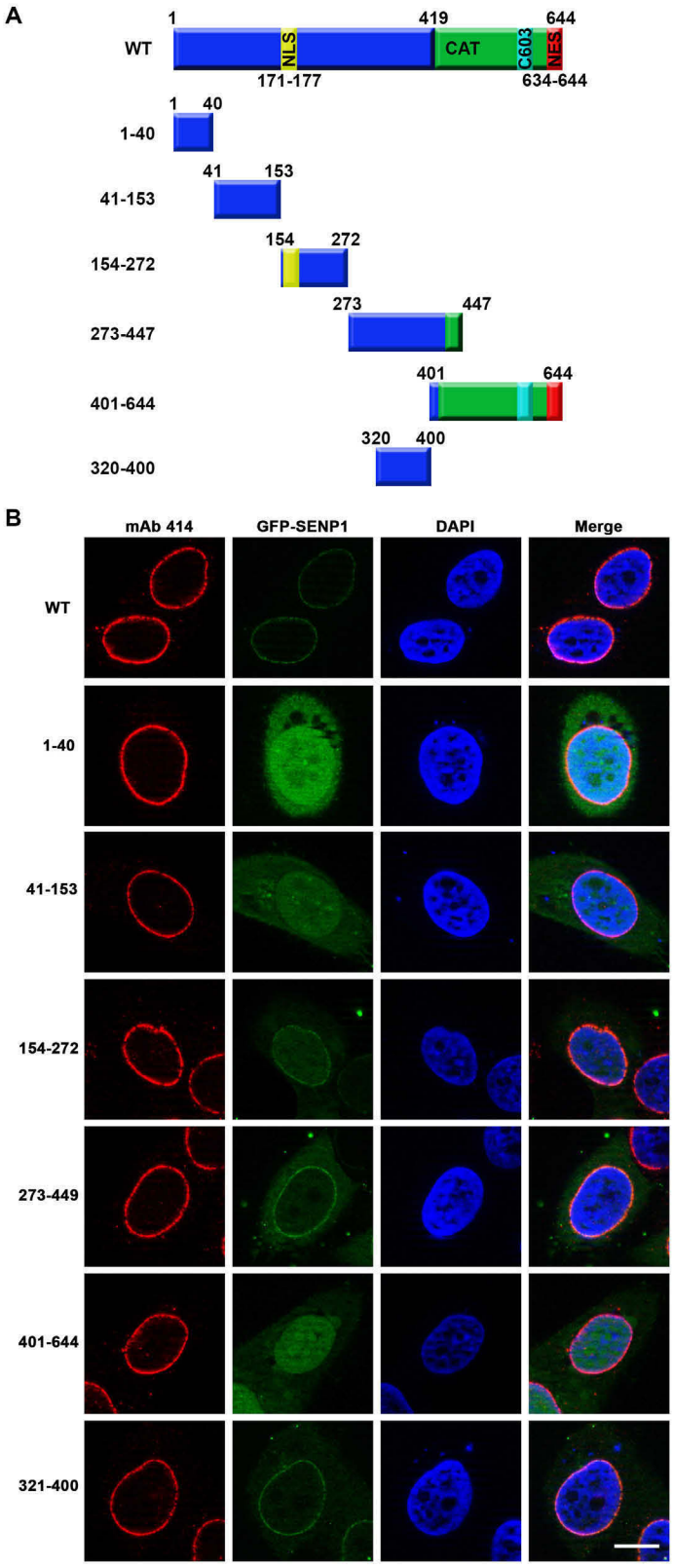

A
WT
1
419
644
NLS
CAT
C603
NES
171-177
634-644
1-40
1
40
41-153
41
153
154-272
154
272
273-447
273
447
401-644
401
644
320-400
320
400
B
mAb 414
GFP-SENP1
DAPI
Merge
WT
1-40
41-153
154-272
273-449
401-644
321-400

Figure 5-3. SENP1 mitotic targeting is mediated by three independent targeting elements. HeLa cells were transfected with GFP-SENP1 full length and the indicated GFP-SENP1 fragments for 48 hours, permeabilized and fixed. Cells were stained with antibodies specific for α-tubulin and CREST and analyzed by immunofluorescence microscopy. DNA is stained with DAPI. Bar = 10 μm.

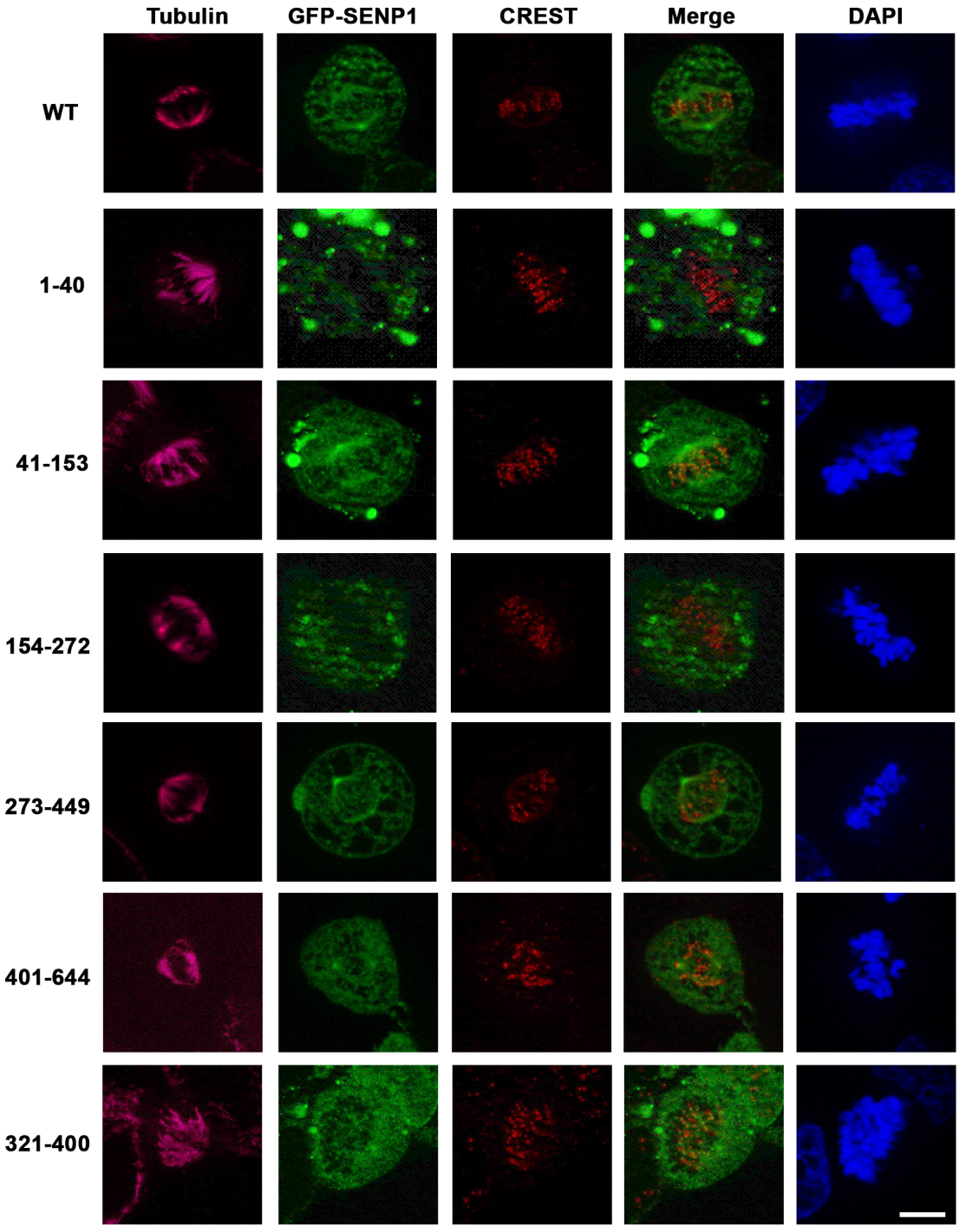

Tubulin
GFP-SENP1
CREST
Merge
DAPI
WT
1-40
41-153
154-272
273-449
401-644
321-400

Figure 5-4. APC activation and sister chromatid cohesion are normal in the absence of SENP1. HeLa cells were transfected with a scramble control or a SENP1-specific oligo for 48 hours. **(A)** Cells were fixed, permeabilized and stained with a cyclin B1 specific antibody. **(B)** Cells were treated overnight with nocodazole prior to harvest and the fraction of transfected cells in mitosis was determined by fluorescence microscopy. Nocodazole synchronization only results in an approximately 30% synchronization after siRNA knockdown **(C)** Cells were fixed in solution, dropped onto slides to spread chromosomes and stained with Geimsa. Chromosomes from cells in multiple states of sister chromatid cohesion are shown. **(D)** Quantification of the sister chromatid cohesion state in scramble control or SENP1 oligo treated cells. Where indicated, error bars represent standard deviations from two independent experiments. DNA was stained with DAPI. Bar = 10 μm.

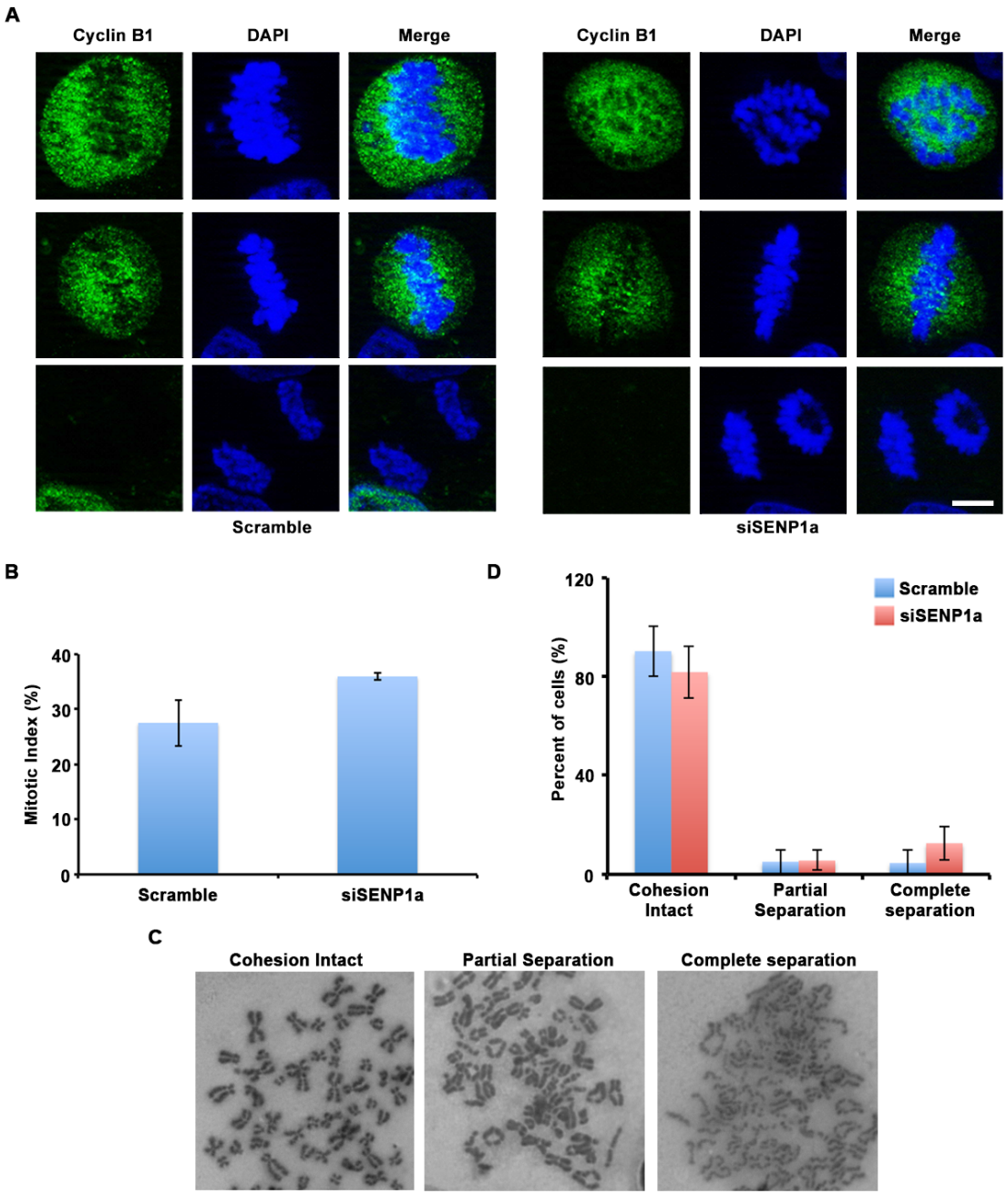

A
Cyclin B1
DAPI
Merge
Cyclin B1
DAPI
Merge
Scramble
siSENP1a
B
Mitotic Index (%)
40
30
20
10
0
Scramble
siSENP1a
D
Percent of cells (%)
120
80
40
0
Scramble
siSENP1a
Cohesion Intact
Partial Separation
Complete separation
C
Cohesion Intact
Partial Separation
Complete separation

Figure 5-5. SENP1 knockdown does not affect mitotic spindle morphology and structure. HeLa cells were transfected with control scramble or one of two SENP1-specific oligos (siSENP1a and siSENP1b) and harvested 48 hours after transfection. **(A)** Cells were fixed, permeabilized and stained with antibodies specific for α-tubulin and CREST to detect the mitotic spindle and centromeres, respectively. **(B)** Cells were treated with nocodazole overnight and washed out for 2 hours prior to harvesting 48 hours after transfection. Cells were fixed, permeabilized and stained with an α-tubulin specific antibody. Spindles in various stages of reformation were observed. **(C)** Cells were fixed, permeabilized and stained with an antibody specific for TRIM28 as a cytoplasmic marker. DNA was stained with DAPI. Bar = 10 μm.

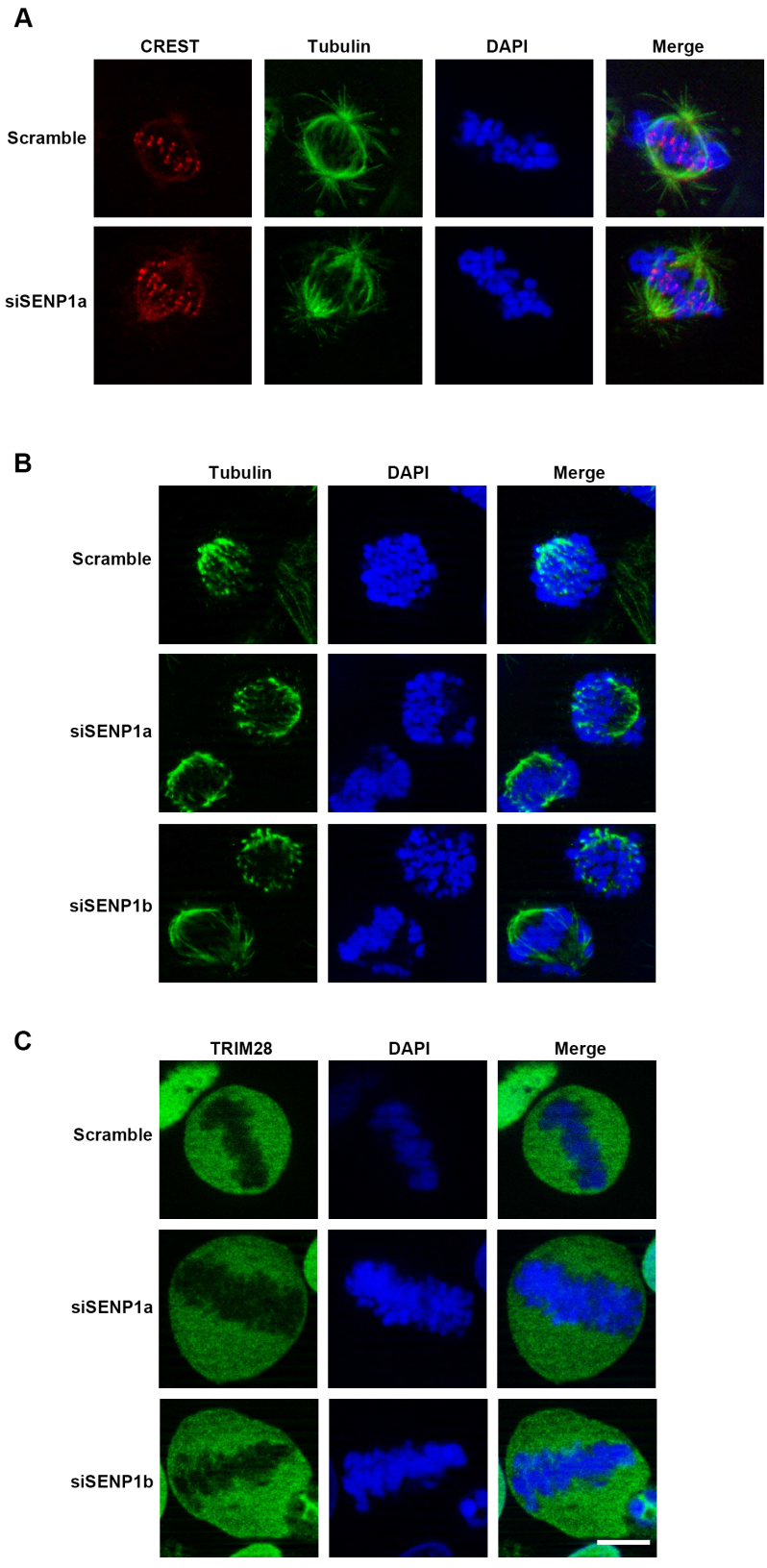

A
CREST
Tubulin
DAPI
Merge
Scramble
siSENP1a
B
Tubulin
DAPI
Merge
Scramble
siSENP1a
siSENP1b
C
TRIM28
DAPI
Merge
Scramble
siSENP1a
siSENP1b

Figure 5-6. SENP1 knockdown does not affect the recruitment and dynamics of centromere and kinetochore proteins. HeLa cells were transfected with a control scramble or a SENP1-specific oligo (siSENP1a) and harvested 48 hours after transfection. Cells were fixed, permeabilized and stained with **(A)** CENP-E **(B)** CENP-F **(C)** CREST **(D)** Hec1 **(E)** INCENP **(F)** Nup358. Pictures were taken at identical exposures for each kinetochore protein in control and siSENP cells.

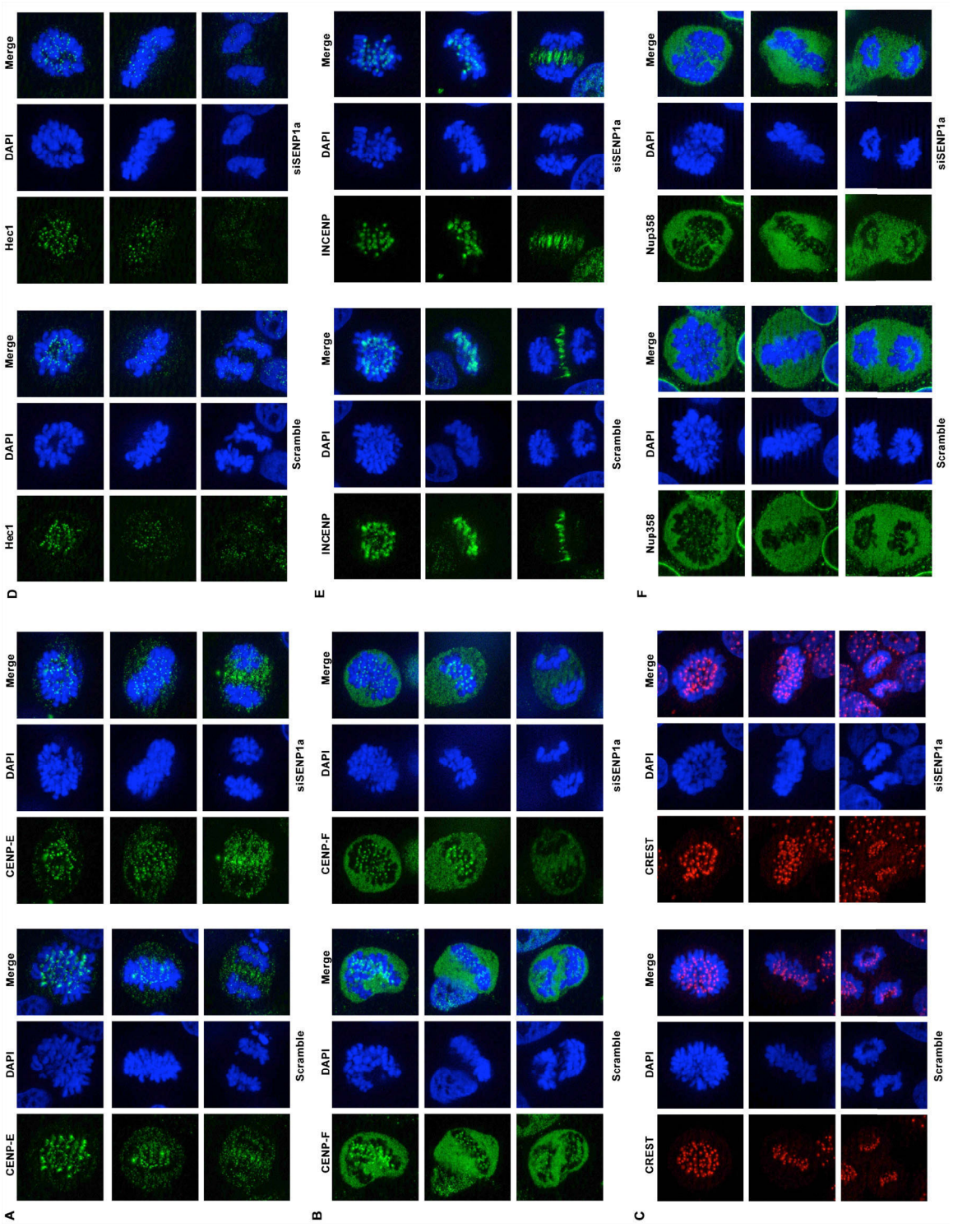

A
CENP-E
DAPI
Merge
Scramble
siSENP1a
B
CENP-F
C
CREST
D
Hec1
E
INCENP
F
Nup358

Figure 5-7. The metaphase to anaphase transition delay after SENP1 depletion is SAC dependent and results in abnormal Mad2 retention at kinetochores. (A) HeLa cells were treated overnight with nocodazole. 1.5 hours prior to harvest, cells were treated with reversine to inactivate the SAC. Mitotic indices were determined by fluorescence microcopy. **(B)** HeLa cells were transfected with control scramble or a SENP1-specific oligo (siSENP1a) and harvested 48 hours after transfection. 1.5 hours prior to harvest, cells were treated with reversine. Mitotic indices were determined by fluorescence microcopy. **(C)** HeLa cells were transfected with control scramble or a SENP1-specific oligo (siSENP1a) and harvested 48 hours after transfection. Cells were fixed, permeabilized, and stained with Mad2-specific antibodies. Arrowheads mark Mad2 retention at kinetochores. DNA is stained with DAPI. Bar = 10 μm.

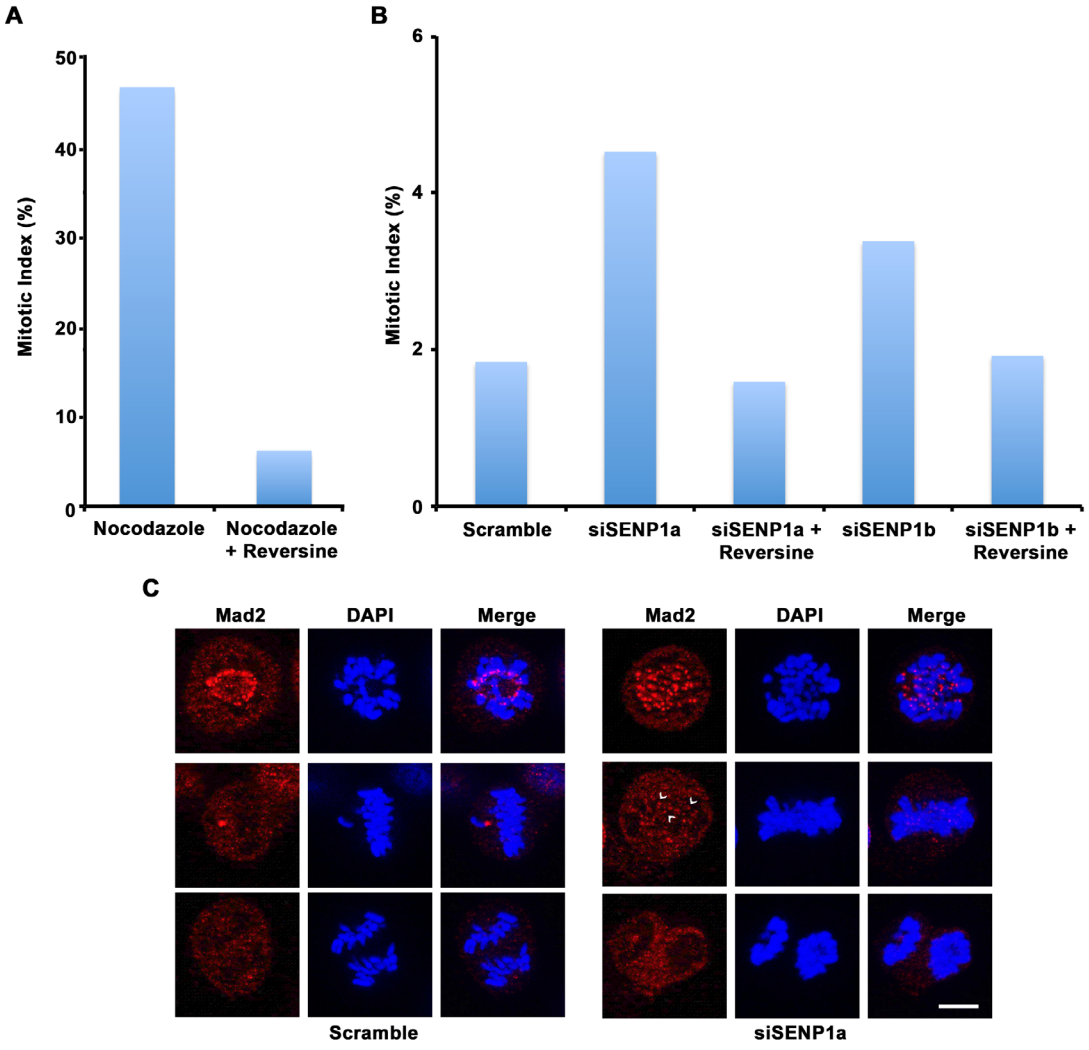

A
Mitotic Index (%)
50
40
30
20
10
0
Nocodazole
Nocodazole + Reversine
B
Mitotic Index (%)
6
4
2
0
Scramble
siSENP1a
siSENP1a + Reversine
siSENP1b
siSENP1b + Reversine
C
Mad2
DAPI
Merge
Mad2
DAPI
Merge
Scramble
siSENP1a

Figure 5-8. SENP1 depletion causes a prolonged S phase. HeLa cells were transfected with a scramble control or one of two SENP1-specific oligos (siSENP1a and siSENP1b) for 48 hours. Cells were trypsinized, fixed in solution and stained with propidium iodide. DNA content was measured with a 488 nm laser on a FACS Calibur flow cytometer. Error bars indicate the standard deviation of three independent experiments. Astericks denote statistically significant results by the student t-test.

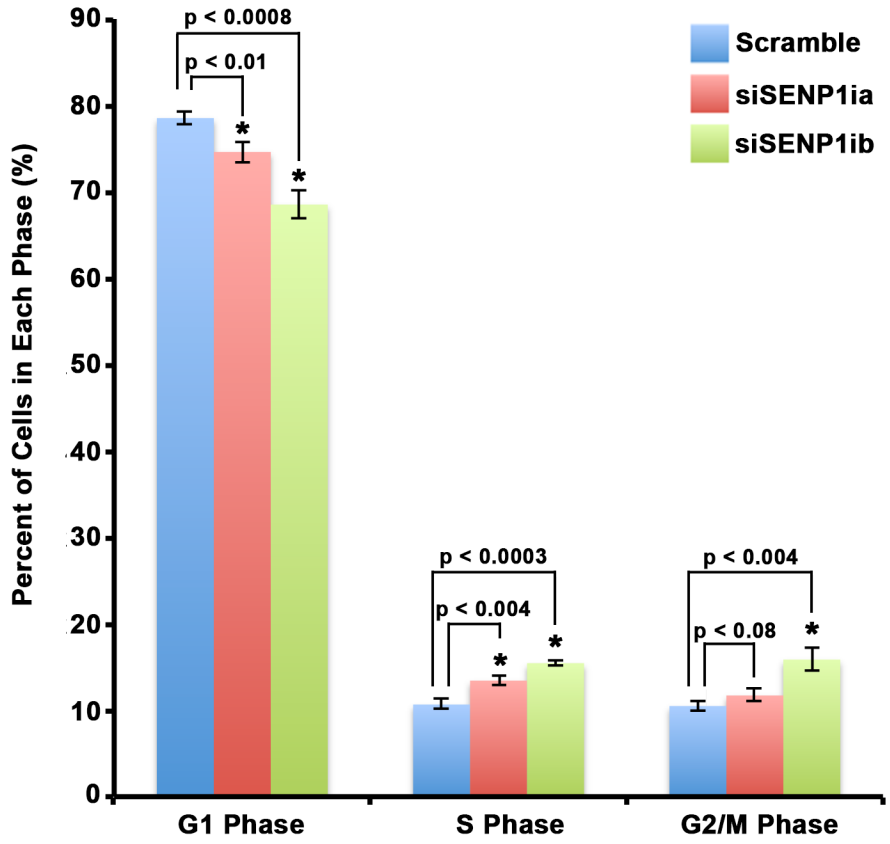
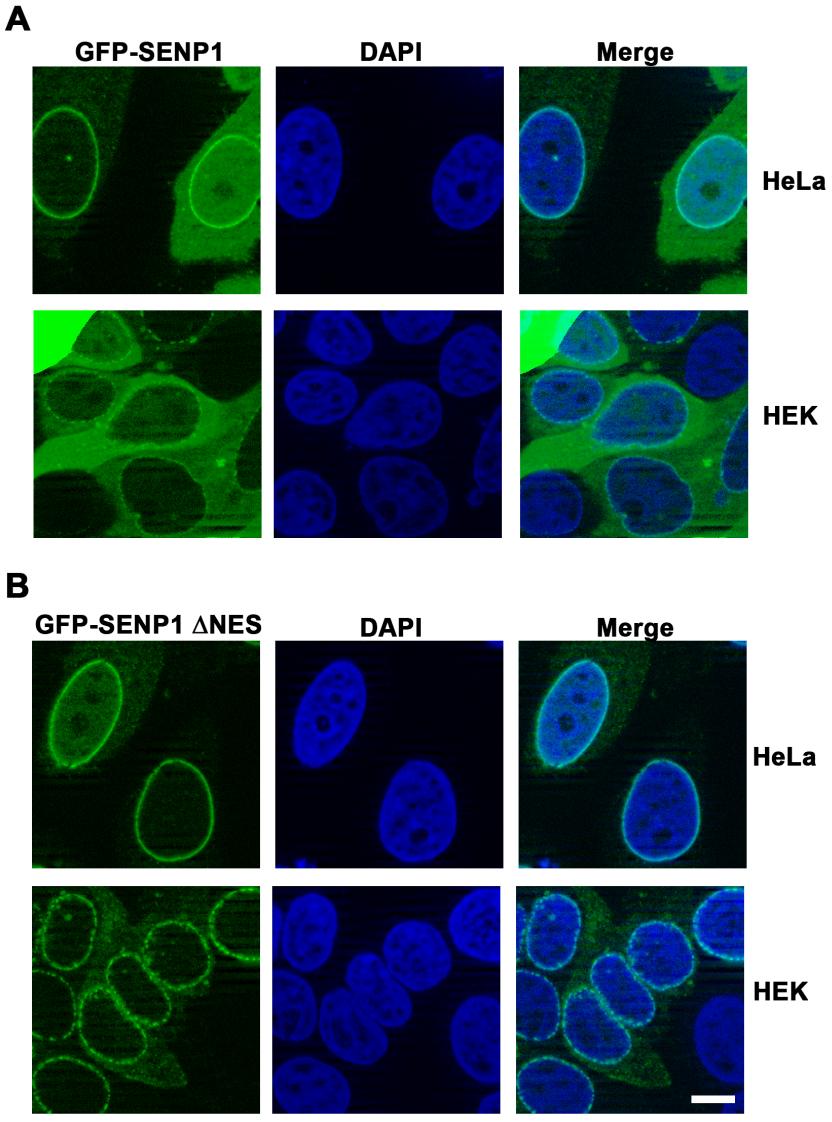

Scramble
siSENP1ia
siSENP1ib
Percent of Cells in Each Phase (%)
90
80
70
60
50
40
30
20
10
0
p < 0.0008
p < 0.01
p < 0.0003
p < 0.004
p < 0.004
p < 0.08
*
G1 Phase
S Phase
G2/M Phase

Figure 5-9. SENP1 cytoplasmic localization in HEK 293T cells is dependent on SENP1 nuclear export. HeLa and HEK293T cells were transfected with **(A)** GFP-SENP1 or **(B)** GFP-SENP1 ΔNES (amino acids 1-633) and harvested 48 hours post transfection. Cells were fixed, permeabilized and visualized by fluorescence microscopy. DNA is stained with DAPI. Bar = 10 μm.


A
GFP-SENP1
DAPI
Merge
HeLa
HEK
B
GFP-SENP1 ΔNES
DAPI
Merge
HeLa
HEK

OVERVIEW AND FUTURE DIRECTIONS

OVERVIEW

Sumoylation was identified as a mitotic regulator more than a decade ago, but the identity of the SUMO-modified proteins and how this sumoylation is regulated in mitosis is still poorly understood (1). In this thesis, we identified the mitotic sumoylated proteins through an unbiased mass spectrometry study. This work will provide a foundation for future characterization of how sumoylation affects the mitotic functions of the identified proteins. In addition, we evaluated the mitotic roles of SENP1 and SENP2 to enhance our understanding of SUMO regulation in mitosis. We utilized SENP2 overexpression studies to demonstrate that sumoylation is required for dynamic association of subcomplexes at the kinetochore and chromosome alignment. Furthermore, we demonstrated that SENP1 isopeptidase activity is required for a timely metaphase to anaphase transition because it is a novel regulator of the spindle assembly checkpoint (SAC). Finally, we conducted a literature review analyzing the functions of sumoylation in regulating chromatin, which may provide insight into the functions of sumoylation at unique chromatin environments like centromeres in mitosis.

CHAPTER 2: Identification of mitotic sumoylated proteins

In this chapter, we conducted a large scale identification of sumoylated proteins associated with mitotic chromosomes. We utilized a combination of nocodazole synchronization, cell fractionation and immunopurification techniques to isolate the SUMO-modified proteins on chromosomes. As a result, we identified 149 proteins that are sumoylated in mitosis. Among these proteins, we identified proteins involved in mitotic progression, chromosome segregation, sister chromatid cohesion, anaphase

promoting complex regulation, mitotic spindle function, and cytokinesis, illustrating the diverse functions of sumoylation in mitosis. Thus, this study provides a foundation for further investigating the molecular mechanisms by which SUMO regulates these diverse mitotic processes.

In addition, we immunopurified and identified SUMO-2/3 modified proteins from G_0 nuclei to determine how many of the mitotic SUMO-modified proteins were also sumoylated at another distinct cell cycle phase. Using this analysis, we identified 89 proteins that were modified in both G_0 and mitosis, which was similar to the degree of overlap between our identified mitotic substrates and proteins identified in other SUMO mass spectrometry analyses. Notably, topoisomerase IIα is among the best characterized mitotic SUMO-modified proteins and its modification is essential for the timely decatenation of the centromeric DNA and sister chromatid separation (2-5). However, our study showed that topoisomerase IIα is sumoylated in G_0 and mitosis, indicating that its regulation through sumoylation is not restricted to mitosis. Thus, it is important to recognize that sumoylation may have functionally important effects on proteins in mitosis, even if those proteins are also modified in other stages of the cell cycle. Determining the functional significance of sumoylation in mitosis must therefore be conducted for each protein of interest, irrespective of whether modification is restricted to mitosis.

Now that a list of chromosome-associated proteins modified by SUMO-2/3 in mitosis has been generated, further analysis needs to be conducted for the substrates of interest. For example, KIF4A was identified as a mitotic sumoylated protein and is known to regulate chromosome condensation and segregation (6). Thus, we need to identify the sumoylation site on KIF4A and make mutant forms that cannot be

sumoylated. Then, we will be able to characterize the functional significance of KIF4A sumoylation in mitosis. Furthermore, it will be interesting to determine if KIF4A sumoylation is uniquely regulated in mitosis. Our preliminary immunoblot analysis demonstrated that more KIF4A was sumoylated in G_0, but our experimental design had many caveats that make a direct comparison of sumoylation levels impossible. Thus, conducting immunopurifications with KIF4A antibodies and then immunoblotting for SUMO, will be critical to evaluating quantitative changes in modification between stages of the cell cycle.

CHAPTER 3: SENP1 and SENP2 regulate mitotic sumoylation

In this chapter, we demonstrated that SENP1 and SENP2 are temporal and spatial regulators of mitotic sumoylation. They both localize to NPCs in interphase and kinetochores in mitosis, but SENP1 has additional mitotic targeting to spindles and centrosomes. However, only SENP2 overexpression caused a chromosome congression defect due to defective recruitment of CENP-E to kinetochores. We determined that this unique mitotic function of SENP2 depends on its interactions with the Nup107-160 subcomplex and karyopherin α3. Furthermore, we showed that SENP1 interacts with the Nup107-160 subcomplex but not karyopherin α3, suggesting that the SENP2 interaction with karyopherin α3 is the distinction between the SENP1 and SENP2 overexpression effects on mitotic progression. Finally, we conducted siRNA knockdown experiments for both isopeptidases but only SENP1 knockdown resulted in a mitotic phenotype. SENP1 depletion caused a delay in the metaphase to anaphase transition, which was rescued by expressing siRNA-resistant WT SENP1 but not the catalytically dead forms. Thus, we

demonstrated that SENP1 and SENP2 have unique and non-redundant functions in regulating mitosis.

To further understand how SENP1 and SENP2 differentially regulate mitosis, it will be critical to identify what substrates each isopeptidase desumoylates. Because SENP-substrate interactions are transient, these interactions are difficult to identify by classical immunopurification techniques. Thus, we have begun to utilize a recently described technique for producing biotin ligase fusion proteins that is very sensitive for detecting protein-protein interactions (7). For these experiments, we will express SENP1 or SENP2 proteins fused to the BirA ligase domain and culture cells with excess biotin. Then, the SENP interacting proteins will be isolated by streptavidin purification and identified by mass spectrometry. Utilizing this proteomic approach, we will identify SENP1 and SENP2 interacting proteins and specifically look for proteins that are bound uniquely by one of the two isopeptidases. Furthermore, if kinetochore proteins are not identified by these methods, we could combine cell synchronization techniques and precisely time the addition of biotin to enrich for proteins that interact with SENP1 and SENP2 during specific cell cycle stages.

CHAPTER 4: SUMO regulates chromatin

In this chapter, we conducted a literature review to illustrate the multi-faceted role of SUMO in regulating chromatin structure. We reviewed evidence that SUMO regulates transcription in a dichotomous fashion, promoting and antagonizing transcription depending on the SUMO substrate. Furthermore, we described the many functions of sumoylation in unique chromatin structures like centromeres, telomeres, and rDNA.

Overall, our study demonstrated that SUMO is critical for many components in chromatin regulation.

The functions of SUMO in interphase chromatin can have profound impacts on mitotic progression. For example, chromosome hypocondensation is observed in MEFs hypomorphic for Ubc9 and in flies lacking a the SUMO E3 ligase Su(var)2-10, indicating sumoylation is required for proper chromosome condensation (8,9). However, chromosome condensation defects can also be caused by problems in chromatin structure (10). Thus, it is unclear if sumoylation is directly regulating chromosome condensation or if the mitotic condensation phenotypes in the absence of sumoylation are merely an consequence of defective chromatin structure. Thus, when investigating mitotic phenotypes related to chromosome condensation or centromere structure, it will be critical to take into account the effects of SUMO on chromatin structure.

In addition, recent work has demonstrated that many chromatin modifying enzymes specifically relocalize to centromeres and kinetochores in mitosis, suggesting potential mitotic functions for chromatin modifiers (11). Furthermore, our mass spectrometry study identified many chromatin remodelers that are sumoylated in both mitosis and G_0. Thus, it is exciting to speculate that sumoylation of chromatin modifying enzymes may regulate their activities in potentially interphase or mitosis. Thus, this review provides a foundation for evaluating and understanding the effects of SUMO regulation on chromatin structure in mitosis and interphase.

APPENDIX: SENP1 regulates SAC activity

In this appendix, we further characterized interactions of SENP1 with the NPC and its functions in mitosis. First, we demonstrated that SENP1 localizes to the nucleoplasmic side of the NPC and that it has two independent NPC-targeting domains. Surprisingly, we demonstrated that SENP1 mitotic localization is mediated by multiple domains that are at least partially unique from SENP1 NPC-targeting. Next, we further characterized the metaphase to anaphase delay observed with SENP1 knockdown. Sister chromatid cohesion, mitotic spindle morphology, kinetochore structure, and activation of the anaphase promoting complex were all normal in the absence of SENP1. Notably, we demonstrated that the metaphase to anaphase delay caused by SENP1 depletion was dependent on the SAC and resulted in abnormal retention of Mad2 at kinetochores, indicating that SENP1 is a novel regulator of SAC function.

To further investigate the mechanism of SENP1 mitotic regulation, we will characterize the interactions between SENP1 and the SAC proteins. Notably, manipulations of dynein, spindly and mps1 have been reported to exhibit similar phenotypes to the SENP1 knockdown, so we will determine if SENP1 regulates their mitotic functions (12-14). Furthermore, we will analyze the sumoylation status of SAC proteins in the presence and absence of SENP1. Notably, BubR1 has already been shown to be sumoylated after long exposures to nocodazole (15). In addition, a more critical analysis of the dynamics of SAC recruitment and release in SENP1-depleted cells will strengthen our conclusion that SENP1 is required for the inactivation of the SAC at anaphase initiation. Finally, Mad1 and Mad2 are SAC proteins that localize to the nuclear

basket just like SENP1 in interphase, so will determine if SENP1 interactions with Mad1, Mad2 or other SAC proteins are mitosis specific or occur throughout the cell cycle.

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EDUCATION

2007-present **PhD, Biochemistry and Molecular Biology (Expected Graduation Fall: 2013)**
Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

2003-2007 **BS, Biochemistry and Molecular Biology; Minor in Spanish**
University of Georgia, Athens, GA
Summa Cum Laude with Highest Honors; First Honor Graduate

RESEARCH EXPERIENCE

2007-present Johns Hopkins University
PhD Research Advisor: Michael J. Matunis
Dissertation: The role of sumoylation in mitotic regulation

2003-2007 University of Georgia
Research Advisors: Marcus Fechheimer and Ruth Furukawa
Project: Investigation of the physiological role of Hirano Bodies
This work was incorporated into an Honors Senior Thesis.

2006 National Institute of Allergy and Infectious Diseases (Summer Internship Program)
Research Advisor: Polly Matzinger
Project: Do dendritic cells need help to secrete IL-12 p75?

2004 Emory University (Summer Undergraduate Research Program)
Research Advisor: Samuel C. Dudley Jr.
Project: Characterization of zetekitoxin, a newly identified saxitoxin analog from the Panamanian Golden Frog

HONORS AND AWARDS

2011 The Elsa Orent Keiles Fellowship in Biochemistry (JHSPH)
Frederick B. Bang Award (JHSPH)

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2007	Joy P. Williams Science Award (UGA Honors Program) Center for Undergraduate Research Scholar (UGA)
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2003-2005	Center for Undergraduate Research Apprentice (UGA)

PUBLICATIONS

Cubeñas-Potts, C., Osula, O., Subramonian, D., Zhang, X-D., Cotter, R.J., and Matunis, M.J. 2013. *Identification of SUMO-2/3 modified proteins associated with mitotic chromosomes.* Submitted to Molecular and Cellular Proteomics. Currently under revision.

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POSTER PRESENTATIONS

SENP2 functions at the kinetochore to regulate chromosome congression.
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NCI Symposium on Chromosome Biology, NIH, Bethesda MD, 2011

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C. Cubeñas Potts, T. Matsusaka, and M.J. Matunis.
The American Society for Cell Biology 50th Annual Meeting, Philadelphia PA, 2010.

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C. Cubeñas Potts, T. Matsusaka, and M.J. Matunis.
NCI Symposium on Chromosome Biology, NIH, Bethesda MD, 2010.

Identification of SUMO-2/3 Substrates in the Late Mitotic Stages.
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Mid-Atlantic Centromere Workshop, NIH, Bethesda MD, 2009.

Studies of Hirano Bodies and Oxidative Stress in Neuroglioma Cells.
R. Furukawa, **C. Cubeñas**, and M. Fechheimer.
The American Society for Cell Biology 46th Annual Meeting, San Diego, CA, 2006.

Characterization of zetekitoxin, a newly identified saxitoxin analog from the Panamanian Golden frog.
C. Cubeñas, A. E. Pfahnl, G. Choudary, and S. C. Dudley Jr.
19th Annual National Conference of Undergraduate Research, Lexington VA, 2005.

LEADERSHIP/MENTORSHIP EXPERIENCE

2010-2011	Trained and mentored Jeremy Vidal, an undergraduate student from JHU, in the laboratory. I trained her on all laboratory techniques and helped design the experiments for her project.
2009	Served as the graduate student representative to the faculty. Attended faculty meetings and helped facilitate communication between the students and faculty.

TEACHING EXPERIENCE

2011-2013	Guest Lecturer, Johns Hopkins University *Principles in Cell Biology* Presented one lecture during the course titled "Mitosis and chromosome segregation."
2011	Teaching Assistant, Johns Hopkins University *Genomics for Public Health (1 quarter)* Wrote and presented two computer laboratory classes which taught students how to use NCBI, BLAST, ClustalW2, and PyMOL; held office hours to meet with students one-on-one; assisted the directors of the course to write and grade an assignment to evaluate student knowledge after these laboratories.

2010	Teaching Assistant, Johns Hopkins University *Introduction to Molecular Biology (1 quarter)* Attended lectures; led a weekly review session to discuss class material and answer questions; held office hours to meet with students one-on-one; assisted the directors of the course to prepare and grade the examinations.
2009-2010	Teaching Assistant, Johns Hopkins University *Principles in Cell Biology (2 quarters)* Attended lectures; led review sessions to discuss class material and answer questions; held office hours to meet with students one-on-one; presented three lectures "Cell Cycle Regulation," "SUMOylation and Post-translational Modifications," and "Mitosis and Chromosome Segregation;" assisted the directors of the course to prepare and grade the examinations.
2005-2006	Teaching Assistant, University of Georgia *Seminar series for students in the Center of Undergraduate Research- Apprentice Program (2 semesters)* Attended all classes; assisted in writing the course syllabus; led small group discussions; presented lecture "How to critically analyze scientific journal articles."

REFERENCES UPON REQUEST